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Investigating the function of *VANGL2* in intestinal homeostasis & disease

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Declaration

I, the undersigned, hereby declare that this thesis is my own composition, the work presented in the thesis has been conducted by myself, unless acknowledged otherwise, and it has not been submitted for any other degree or professional qualification except as specified.

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Abstract

Introduction: Van Gogh-Like 2 (*VANGL2*) is a scaffolding planar cell polarity protein involved in non-canonical Wnt signalling. It has been shown to have crucial roles in regulating epithelial development and homeostasis. Moreover, *VANGL2* has been implicated in human cancers, with increased expression and copy number amplification seen in several cancer contexts. Many related components within this pathway have also been linked to cancer development, with *VANGL2* expression known to regulate factors involved in cell migration and extracellular matrix (ECM) remodelling in cell lines. These cellular processes tend to be erroneously activated in cancer. *VANGL2* is known to inhibit the classical driver pathway of colorectal cancer (CRC), canonical, or β-catenin dependant, Wnt signalling, in CRC cell lines. The aim of this thesis is to determine the expression of *VANGL2* in CRC, and to investigate how *VANGL2* may act to regulate intestinal homeostasis and disease.

Methods: Transcriptional verification of *VANGL2* expression in the mouse intestine was carried out by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), and transcripts localised within the murine colon using RNA-In Situ Hybridisation (RNA-ISH). Expression and localisation of the *VANGL2* protein and related non-canonical

Wnt signalling components was confirmed using immunohistochemistry (IHC). Furthermore, using a combination of human Tissue Micro-Array (TMA), transcriptional data and genomic data, we determined an association between *VANGL2* on tumour grade and disease-free survival.

To functionally validate the effects of *VANGL2* on colorectal biology, we used a model in which *VANGL2* is selectively deleted from the colonic epithelium using *Villin-Cre*^{ERT} *Vangl2*^{flox} mouse lines. Using a combination of molecular biology methods, we identified the ECM as differentially regulated following *VANGL2* modulation.

To test the role of *VANGL2* in colorectal cancer, we used a murine colorectal cancer model in which adenomatous polyposis coli (APC) is deleted from colonic epithelium resulting in the formation of cancer concurrently with deletion of *Vangl2*. We evaluated survival of these mice as well as tumour number and size. Tumour tissue was analysed using IHC, qRT-PCR and 3-Dimensional organoid culture.

Results: Within this thesis I have illustrated that the murine colonic epithelium expresses *Vangl2*, and other components known to interact with *VANGL2* including *Vangl1*, Wnt5A, and Protein Tyrosine Kinase 7 (*Ptk7*). I have also shown that *VANGL2*

is expressed within the human colonic epithelium. I go on to show that 9.2% of human CRC possesses *VANGL2* transcriptional alterations which correlates with a worsened disease-free survival (DFS) rate among patients. Using IHC, I also show that higher grade CRC is associated with increased *VANGL2* expression.

In our murine cancer model, mice with single or dual-copy loss of *VANGL2* were found to have a reduced number of colonic tumours, while maintaining similar tumour size. Investigations to identify how *VANGL2* may have control of tumour initiation were carried out focussing on the ECM. I found that, contrary to what I have discovered in the healthy murine colon, tumours from *VANGL2*-deficient mice had increased transcription of the ECM markers Secreted protein acidic and rich in cysteine (*Sparc)* and Decorin (*Dcn)*, as well as increased expression of the ECM regulators Matrix Metallopeptidase 9 (*Mmp9)* and Tissue Inhibitor of Metalloproteinases 1 (*Timp1)*. Changes in the ECM was also seen at the protein level, with increases in staining for the ECM components *Col1* (Collagen, type I), and *Laminin* in *VANGL2*-deficient tissue. The ECM modulator Connective Tissue Growth Factor (*Ctgf)*, is implicated in multiple cancers including CRC and is increased within *VANGL2*-deficient tumours at both the transcript and protein level, implicating *Ctgf* in increasing the ECM of these tumours.

Conclusion:

VANGL2 is expressed within the human colonic epithelium and its transcriptional or genomic alteration is associated with poorer DFS in human CRC, while higher grade tumours show increased *VANGL2* marker staining. The murine colon epithelium expresses *VANGL2* and related non-canonical Wnt signalling components. Loss of *VANGL2* in the colonic epithelium leads to alteration of downstream Wnt target expression, and of ECM component expression. Loss of *VANGL2* reduces the number of tumours developed in our intestinal cancer model while not altering tumour size. *VANGL2*-deficient tumours have increased ECM component expression and increased CTGF expression.

Lay Summary

Although many patients survive bowel cancer, the chances of survival remain relatively low should the disease be diagnosed at an advanced stage, or occur in an aggressive form. One of the main ways bowel cancer forms is through changes in a signal which controls how bowel cells behave, known as Wnt signalling, which causes cells to grow more quickly.

While there are parts of this Wnt signalling that we understand, there are other aspects that remain a 'black box'. In this thesis, I have studied this black box, concentrating on a Wnt gene known as *VANGL2*. It has been suggested that changes in this gene play a part in bowel cancer and I wanted to better understand how *VANGL2* functions in colonic health and disease.

I show that *VANGL2* is present in human bowel cells, and I have found that if you have more *VANGL2* in tumours there is increased risk of cancer recurrence following treatment. Using mouse models to change how *VANGL2* functions, I found that the Wnt signalling pathway that *VANGL2* regulates was altered. Importantly, this pathway regulates the ECM, a net of proteins that holds cells together and which is needed for colonic cells to behave normally. By identifying

that *VANGL2* can change how cells in the colon interact with this net we will be able to design drugs that regulate this process and have the potential to help improve colon repair in colitis or prevent bowel cancer growth.

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Abbreviations

AP-1 – Activator Protein 1
APC – Adenomatous Polyposis Coli
BMP4 – Bone Morphogenetic Protein 4
BSA – Bovine Serum Albumin
CAMKII – Calcium/calmodulin-dependent Kinase II
CBC – Crypt Base Columnar
COL1 – Type I Collagen
CRC – Colorectal Cancer
CTGF – Connective Tissue Growth Factor
DAB – Diaminobenzidine
DCN – Decorin
DFS – Disease-free Survival
DKK1 – Dickkopf1
DVL – Dishevelled
ECM – Extracellular Matrix
EDTA – Ethylenediaminetetraacetic acid
EGF – Epidermal Growth Factor
ER – Endoplasmic Reticulum
FAP – Familial Adenomatous Polyposis
FN – Fibronectin
FZD – Frizzled
GFP/RFP – Green/Red Fluorescent Protein
HNPCC – Hereditary Nonpolyposis Colorectal Cancer
IBD – Inflammatory Bowel Disease
IHC – Immunohistochemistry
JNK – C-Jun NH2-terminal Kinase
LOF – Loss-of-function
LRP5/6 – Low-density Lipoprotein Receptor-related Protein 5/6
MAP – MUTYH-associated polyposis
MMP – Matrix Metallopeptidase
MSI – Microsatellite instability
MSS – Microsatellite Stable
NSAID – Non-steroidal Anti-inflammatory Drug
NTD – Neural Tube Defect
OS – Overall Survival
PBS – Phosphate-buffered Saline
PCP – Planar Cell Polarity
PKC – Protein Kinase C
PORCN – Porcupine
PTK7 – Protein Tyrosine Kinase 7
qRT-PCR – Quantitative Real-time Polymerase Chain Reaction
QS – Quickscore
RNA-ISH – RNA In-Situ Hybridisation
ROCK – Rho-associated Protein Kinase
ROR2 - receptor tyrosine kinase like orphan receptor 2
SC – Stem Cell
sFRP – Secreted Frizzled-related Protein
shRNA – Short-hairpin RNA
siRNA – Short-interfering RNA
SPARC – Secreted Protein Acidic and Rich in Cysteine
TA – Transit Amplifying
TGF-β – Transforming Growth Factor- β
TIMP – Tissue Inhibitor of Metalloproteinases
TMA – Tissue Microarray
VANGL1 – Van-Gogh-like 1
VANGL2 – Van-Gogh-like 2
VEGF – Vascular Endothelial Growth Factor
YAP/TAZ – Yes-associated Protein/Transcriptional Coactivator with PDZ-binding Motif
αSMA – A-smooth Muscle Actin

Chapter 1: Introduction

Colorectal cancers (CRC) are the third most prevalent cancer in Scotland, with 3,671 cases diagnosed in 2015. This represents a 6% lifetime risk that a person will develop CRC. As well as being a cancer with a high rate-of-incidence, it is one of the deadliest, being the second-highest in terms of cancer-related mortality, with 1,565 deaths from CRC per year in Scotland (10% of all cancer mortality). CRC mortality is only surpassed by lung cancer (ISD, 2016).

In the period of 2005-2015, there have been improvements in survival. During this time, CRC-related mortality had decreased an average of 16% (21% for men and 8% in women). Survival rates of patients with CRC have also seen considerable improvement. Patients diagnosed between 1987-1991 had a five-year survival rate of 35.9%, however patients diagnosed between 2007-2011 have a 59.5% five-year survival rate. This is due to earlier diagnosis and to improvements in treatment (Bujanda et al., 2010). This said, improving patient CRC survival with small-molecule or biological therapies, targeted at specific cell signalling pathways, has yet to be realised. One of the most important cellular pathways in CRC development is the Wnt signalling pathway.

The Wnt signalling network is composed of signal transduction pathways that are dependent on the Wnt ligand, a small glycoprotein which is secreted from cells and initiates a number of signalling cascades. The Wnt signalling network consists of canonical and non-canonical Wnt pathways. Together these signals control several critical physiological functions such as self-renewal, cell fate determination, proliferation, polarity, and organogenesis. It has been demonstrated to be critical in adult tissue homeostasis as well as in the establishment of proximal-distal (or planar) polarity in drosophila organ development (Baena-López, Baonza, & García-Bellido, 2005; Fevr, Robine, Louvard, & Huelsken, 2007), highlighting this pathway as a highly conserved signalling cascade, central to multicellular life. As well as this role in adult tissue maintenance and development, loss of control of Wnt signalling in adult homeostasis can result in diseases such as CRC and other cancers.

1.1 Colorectal Cancer

Colorectal cancer, also commonly known as bowel cancer or colon cancer, falls under the ICD-10 (international classification of diseases) C18-C20. It is cancer of the colon or rectum in the large intestine (or large bowel). CRC originates from epithelial cells, which line the lumen of the colon or rectum. Uncontrolled growth of these cells can result in a polyp, or an adenoma. These are usually protrusions of tissue into the lumen of the gut.

Aberrant crypt foci (ACF) have been proposed as the initial stage of colorectal adenoma development, and the precursor to the development of an adenoma. These were first identified in 1987 by Bird et al in rodent models of carcinoma, and later shown in the human colonic epithelium. ACF normally appear with a 'slit'-like crypt lumen shape, increased crypt size, increased epithelial proliferation, and these lesions are associated with K-ras mutations (Bird, 1987, 1995; Pretlow, Brasitus, Fulton, Cheyer, & Kaplan, 1993; Pretlow, O'Riordan, Spancake, & Pretlow, 1993; Stellato et al., 1991)

Adenomas, or adenomatous polyps, are frequently found in humans over 50 years of age, with ~40% of patients having ≥1 adenoma (Winawer et al., 1993).

Adenomas are typically described as being tubular, tubulovillous, or villous, with the majority occurring as tubular. Adenomas that grow to larger than 2 cm in diameter are associated with containing malignant cells, as this size of tumour requires vascularisation to survive. Adenomas tend to grow slowly, often over a period of years. As the adenoma grows, the risk of the adenoma acquiring malignant characteristics and becoming an adenocarcinoma, increases. Adenocarcinomas possess the ability to invade into the adjacent colonic tissue and breach the submucosa (wall of the colon), which is known as invasion. Many adenomas can be removed via colonoscopy very easily, however, if allowed to become an adenocarcinoma and invade into the submucosa, treatment becomes much more difficult. For this reason, during a colonoscopy all discovered polyps will be removed if possible.

If an adenocarcinoma is permitted to develop further, it may invade adjacent organs or the visceral peritoneum. Further progression may lead to metastasis by spread of malignant cells to regional lymph nodes or distant organs/lymph nodes. This often occurs through the shedding of cells into the circulatory system. Multiple genetic alterations, discussed below, are necessary for normal colorectal epithelium to become cancerous. The most common organs to find metastasis in CRC are the liver and lung. Also, metastasis can be found in the peritoneum.

1.1.1 Risk Factors for CRC

Increasing age is the highest risk factor for colorectal cancer. This is mainly because, due to imperfect DNA replication, cells accumulate mutations with age, and an increasing number of mutations increases the likelihood that a tumour-regulating pathway disturbed, this is particularly problematic in tissues with a high rate of turnover, where constant replication of genetic material is required in order to maintain homeostasis (Tomasetti & Vogelstein, 2015). Other risk factors include a family history of colorectal cancer, personal history of colorectal adenomas/cancer or ovarian cancer, hereditary conditions such as familial adenomatous polyposis (FAP) and Lynch syndrome (hereditary nonpolyposis colorectal cancer [HNPCC]) and personal history of chronic ulcerative colitis or Crohn's. Environmental factors such as excessive alcohol use, cigarette smoke and obesity also lead to increased risk of colorectal cancer disease (Fedirko et al., 2011; Imperiale et al., 2014; Johns & Houlston, 2001; Laukoetter et al., 2011; P. S. Liang, Chen, & Giovannucci, 2009; Ma et al., 2013; Mork et al., 2015; PDQ Adult Treatment Editorial Board, 2002; Singh, Nugent, Demers, Czaykowski, & Mahmud, 2013; Srinivasan, Yang, Rubin, Morgan, & Lewis, 2007). Both the biological factors and epidemiology for each major risk factor is described in more detail below.

1.1.1.1 History of Colorectal cancer or intestinal diseases

Patients who have a history of adenomas also have an increased risk of developing CRC (De Jong et al., 2005). Similarly, patients with a family history of CRC or adenomas have a significantly increased risk of developing CRC over those patients without such family history. This risk is greater for those with relatives diagnosed before the age of 45 or with 2 or more affected relatives (Johns & Houlston, 2001), suggesting that there are potential poorly defined genetic susceptibilities which have not been elucidated, or that there are local environmental factors which confer this increased risk. Whilst risk of CRC development is certainly transmitted through families, there are several hereditary colorectal cancer conditions which have been linked to causative variants being passed longitudinally from parents to their probands. Lynch syndrome, FAP, MUTYH-associated polyposis (MAP) and attenuated FAP (AFAP) are all inherited and carry a lifetime risk of colorectal cancer of between 50-100% (Leoz, Carballal, Moreira, Ocaña, & Balaguer, 2015). Conditions which confer a high CRC risk are summarised in Table 1.1. Inflammatory bowel disease (IBD), which includes both Crohn's disease and ulcerative colitis, may cause patients to have an increased risk of colorectal cancer. However, evidence in this area is conflicting around the actual level of increased risk. This is possibly due to patients with IBDs receiving greater endoscopic surveillance, colectomies, and therapies which reduce the incidence of CRC (Triantafillidis, Nasioulas, & Kosmidis, 2009).

Condition	Inheritance	Gene(s)	Lifetime CRC Risk	Adenoma Number	Polyposis
Lynch syndrome (HNPCC)	Autosomal dominant	hMLH1, hMLH2, hMSH6, hPMS2	50-80%	= general population	No
Familial adenomatous polyposis (FAP)	Autosomal dominant	APC	100%	100-1000s	Yes
Attenuated FAP	Autosomal dominant	APC	70%	<100	Yes
MUTYH-associated polyposis	Autosomal recessive	MUTYH	80%	<100	Yes
Peutz-Jeghers syndrome	Autosomal dominant	STK11	39%	> general population	Yes
Juvenile polyposis syndrome	Autosomal dominant	SMAD4, BMPR1A	39%	> general population	Yes

Table 1.1 Characteristics of high-risk colorectal cancer conditions. Adapted from Hereditary and Familial Colon Cancer (Can, Kaymaz, Pochi, & Aktimur, 2013).

1.1.1.2 Environmental risk factors

A meta-analysis by Fedirko et al found that moderate (2-3 drinks/day) and heavy (≥4 drinks/day) consumption of alcohol had an increased risk of colorectal cancer of 21% and 52%, respectively. Males and Asian participants were found to particularly susceptible to this association. These correlations were found to be dose dependent, as doses of alcohol at 10, 50 and 100 g/day were found to increase the chance of colorectal cancer at 7%, 38% and 82%, respectively (Fedirko et al., 2011).

In addition, Liang, Chen and Giovannuci found that current and former smokers had a significantly increased risk of CRC incidence and mortality relative to non-smokers. Specifically, an increase of 40 cigarettes per day causes a 38% increase in risk of developing CRC. Over a 40-year period of smoking there is a 20% increase in risk. The same study also found that 60 pack years led to a 51% increase in CRC risk (a pack year is defined as twenty cigarettes smoked every day for one year) (P. S. Liang et al., 2009).

As well as alcohol and cigarette smoke, obesity has been implicated in increasing the lifetime risk of CRC. Ma and colleagues found through meta-analysis, that individuals within the obese category of body mass index (BMI) have a 33%

higher chance of CRC than those within the normal category. Those within the highest category of waist circumference were found to have a 46% higher risk than those within the lowest category (Ma et al., 2013). In support of this, another analysis found for every 5 kg of weight gain, the risk of CRC increased by 4% (Schlesinger et al., 2015). Furthermore, dietary composition could have an impact on the likelihood of developing CRC, with red meat being a controversial risk factor in CRC development. The potential impact of red meat consumption on CRC risk is widely contested. A study by Xu et al found that a 50 g/day increase in processed meat consumption resulted in a 28% increased risk, and that a 100 g/day increase in red meat intake increased the risk of colorectal adenomas by 36% (Xu et al. 2013). In a meta-analysis, it was found that high red meat consumption was associated with an 11% increased risk of CRC. However, this risk reduced when the meta-analysis was restricted to studies that considered only fresh red meat specifically. As well as this, no clear dose-response emerged from red meat intake and CRC (Alexander, Weed, Miller, & Mohamed, 2015).

Conversely, Terry and colleagues, using data from a Swedish female cohort, found an inverse correlation between total fruit and vegetable consumption and CRC. Individuals who consumed less than 1.5 servings of fruit and vegetables per day had an increased risk of 65% compared to those who consumed more than 2.5 servings. They also found that higher consumption of cereal fibre did not lower the risk of

colorectal cancer (Terry et al., 2001). A meta-analysis performed in 2002 specifically looked at dietary fibre, and found no statistically or clinically significant association with incidence or recurrence of adenomatous polyps (Asano & McLeod, 2002).

While diet has been closely studied as a causative factor in CRC, use of non-steroidal anti-inflammatory drugs (NSAIDs) has also been inversely correlated with CRC. Din and colleagues found that low-dose aspirin use (75 mg daily) associated with a decreased CRC risk of 22% in a Scottish cohort. Similarly, non-aspirin NSAIDs were also found to have a protective effect against CRC. No effect on CRC survival was found as a result of aspirin/NSAID intake (Din et al., 2010). Furthermore, Rostom et al found CRC incidence was reduced by 30-39% with use of non-aspirin NSAIDs, but no association with CRC survival was found (Rostom et al., 2007). Using a large cohort (301,240 individuals), Ruder et al demonstrated that the use of aspirin and non-aspirin NSAIDs lowered CRC risk by 9% and 18%, respectively, when compared to non-users of these drugs (Ruder et al., 2011). These data show that aspirin and other NSAIDs can be protective against CRC incidence when taken in the right contexts, however whether NSAIDS should be used prophylactically remains to be seen given the potential off-target effects of these drugs.

Finally, the link between physical activity and colorectal cancer risk has also been investigated. A meta-analysis by Shaw and colleagues found that the association between physical activity and colorectal cancer risk for those with a family history of colorectal cancer and those without was 0.72 and 0.56, respectively (Shaw et al., 2018). This inverse correlation of physical activity with colorectal cancer risk indicates that it may be useful as a cancer prevention strategy.

Studies have gone some way to showing genetic-environmental interactions in CRC risk. For example, Hutters *et al* examined potential effect-modification of previously identified CRC susceptibility loci and major CRC environmental risk factors (sex, body mass index, height, smoking status, aspirin/NSAID use, alcohol use, as well as calcium, folate, red meat, processed meat, vegetables, fruit, and fibre intake) (Hutter et al., 2012). The strongest positive interaction was found between vegetable consumption and the SNP rs16892766 (chromosome 8q23.3; near the EIF3H and UTP23 genes). In a genome-wide investigation of the genetic interaction with obesity, smoking, and alcohol consumption in CRC risk, obesity was found to have a statistically significant negative interaction with the SNP rs1944511 (Siegert et al., 2013).

1.1.2 CRC Prognostic Markers

Prognosis for patients with CRC is based on several factors: 1) the extent of invasion of tumour into the wall of the colon, 2) the presence or absence of cancerous cells in lymph nodes, and 3) the presence or absence of distant metastases. The TNM classification of malignant tumours, the most widely used cancer staging system worldwide, is used to reflect the features that characterise a patient's cancer. 'T' represents the primary tumour. A score of 0-4 is assigned to represent the size and extent of the invasion by the tumour. 'N' represents lymph node involvement. A score is given representing the number of lymph nodes that contain cancer. Distant metastases are represented by 'M'. A score of zero means there are no metastases detectable and a score of one indicates a metastasis is present (National cancer institute, 2017). The TNM classification can be more broadly represented by the AJCC (American Joint Committee on Cancer) stage (AJCC, 2018). This progresses from stage 0 to Stage IV. Stage 0 represents a T0 stage cancer (cancer confined to mucosa), stage I represents invasive T1/T2 tumours. Stage II cancers are typically T3/T4, with the tumour invading adjacent tissues. Stage III cancers involve metastasis to regional lymph nodes, and are often N1/N2. Stage IV cancers possess distant metastases, which are M1 by TNM definition.

Additionally colonic tumours can be graded histologically using a score of G1-G4, with G1 meaning that the tumour is well-differentiated (i.e. the tumour has normal tissue structure) and with G4 meaning the tumour is undifferentiated (tumour has high architectural abnormality, meaning the cells do not look like the cells from which the tumour arose) (National Cancer Institute, 2013).

Other pathological prognostic features include the presence of signet ring-cell carcinoma. This is defined by most tumour cells possessing prominent intracytoplasmic mucin, which occurs with nuclear displacement within the cell. Patients with signet-ring cell carcinoma (around 1% of CRCs) have reduced five-year survival when compared to other subsets of colorectal carcinoma (Kang, O'Connell, Maggard, Sack, & Ko, 2005). Mucinous adenocarcinoma, which is defined as the majority of the tumour is composed of extracellular mucin represents 12% of CRCs (Langner et al., 2012). While survival between all stages of mucinous and non-mucinous adenocarcinomas are not significantly different, when stage III carcinomas are considered patients with mucinous adenocarcinomas have worsened survival (Xie, Villeneuve, & Shaw, 2009). As well as this, Betge and colleagues identified both venous and lymphatic invasion as significant prognostic variables in patients with CRC, associating with tumour classification, lymph node status, and CRC stage (AJCC) (Betge et al., 2012).

Several other factors can be used as prognostic markers for CRC, such as the presence of a bowel obstruction or perforation (Steinberg, Barkin, Kaplan, & Stablein, 1986). Novel, circulating biomarkers are also being identified to help detect CRC in a less invasive way.

1.1.3 CRC Management

DIAGNOSIS: Symptoms suggestive of CRC include changes in bowel habits, rectal bleeding, and bloody stool. The primary method of colorectal cancer investigation is via colonoscopy, however small lesions can be missed (Leslie & Steele, 2002).

SCREENING: Patients considered to have high-risk for CRC are given regular colonoscopic surveillance. High-risk patients include those with a family history of CRC (including those with hereditary CRC conditions), those with a personal history of adenomatous polyps and/or colorectal cancer, and those with inflammatory bowel disease (IBD). For the general population, faecal occult blood testing is used for those over 60 years of age. A meta-analysis of this type of screening by Bernie *et al* found it led to a reduction in mortality by 23% (relative risk 0.77 (0.57 to 0.89) for those who undertake the screening (Towler et al., 1998). The same study found that one-third of those invited did not participate, thereby limiting the effectiveness of this type of screening.

TREATMENT: Current standards of care for CRC are as follows: stage 0 colorectal cancer is usually treated with polyp removal during a colonoscopy. Stage

I is treated with surgical removal of the tumour. Stage II colorectal cancer is usually treated with surgical resection of the tumour, with optional adjuvant chemotherapy. Stage III colorectal cancer is treated with surgical tumour and associated lymph node removal, combined with adjuvant chemotherapy. Stage IV colorectal cancer treatment is often more variable, depending on which tissues the cancer has spread to. Often the treatment plan would include a combination of surgical resection, chemotherapy, and radiotherapy. Recurrent CRC is often given a treatment plan similar to stage IV (Cancer.net, 2017).

1.1.4 The Intestine

The mammalian intestine is made up of a mesenchymal smooth muscle lumen, and a luminal lining of a single layer of epithelial cells. There are several distinct anatomical areas within the small intestine (duodenum, jejunum, and the ileum), and large intestine (caecum and the colon). The epithelial surface is invaginated through the mesenchyme to give the morphological structures of the crypts and villi (in the large intestine the invaginations have less depth, and do not form villi). The epithelium of the intestine represents one of the most important barriers in the body. It must be selectively permeable, allowing in nutrients while staying resilient to toxins, foreign antigens, and gut microbiota. A series of protein-protein interactions across cellular junctions in the epithelium, and physical barriers such as mucus allow this barrier to exclude such insults, such as bacteria (Johansson, Sjövall, & Hansson, 2013).

The cells in the intestinal epithelium are continually replaced throughout life, with the entire epithelium is replaced every 3-5 days in the small intestine, and every 5-7 days in the colon (Barker, 2013). This persistent proliferation takes place within the crypt compartments (Figure 1.1). There are two models for the precise identity of the intestinal stem cells (SCs). The SC zone model postulates that crypt base

columnar (CBC) cells present at the bottom of crypts provide the SC pool for epithelial regeneration. In support of this hypothesis, cloning marking techniques show these cells' ability to give rise to multiple cell types in the small intestinal crypt (M. Bjerknes & Cheng, 1999; Matthew Bjerknes & Cheng, 1981). The +4-position model hypothesises that a 'band' of proliferating cells sit in the crypt above non-cycling Paneth cells present in the base. The intestinal SCs are +4 cells from the crypt bottom. Again in the small intestine, Potten et al found that these +4 SCs retain DNA labels for extended periods of time, suggesting limited cycling activity (Potten, Kovacs, & Hamilton, 1974). Barker et al, in pioneering murine lineage tracing work demonstrated that *Lgr5*$^{+}$ CBCs can generate all epithelial lineages over a 60-day period in both the small intestine and colon (Barker et al., 2007).

While cycling, label retaining, stem cells have been shown to act as a key progenitor pool for the epithelium, research by Buczacki et al found that intestinal quiescent cells are precursors to multiple epithelial cell types too, and that in intestinal regeneration following injury they can proliferate and provide the main epithelial cell types (Buczacki et al., 2013). Therefore, there are potentially multiple pools of progenitors within the intestine that can repopulate the epithelium following injury and some may have context-dependent activation. At the bottom of the small intestinal crypts stem cells (identified by the presence of Lgr5) are interspaced by larger, Paneth cells. These long-lived cells contribute to the intestinal barrier by

secretion of anti-microbial products such as defensins. These Paneth cells also supply the epithelial niche for the intestinal stem cells with Wnt3, EGF and Notch ligand (Sato, van Es, et al., 2011). While there are no Paneth cells in the colon, it has recently been reported that $Reg4^{+}$ expressing CBC cells are the Paneth-equivalent cell-type and function as the epithelial niche in the colon (Sasaki et al., 2016)

Wnt signalling plays a crucial role in the maintenance and activation of SC pools, and it is no different in the intestinal stem cell context. For example, Clevers and colleagues found that depletion of one of the main effector molecules of the Wnt signalling cascade, TCF-4 (Transcription factor-4), leads to the loss of proliferative compartments in the murine small intestine. This impairment leads to death shortly after birth. It has also been shown that the intracellular signal transducer, β-catenin, accumulates in the nucleus of intestinal crypt progenitors, which indicates active Wnt signalling.

β-catenin, the defining molecule of canonical Wnt signalling, is accumulated in progenitors in the intestinal crypt. Disruption of β-catenin activity in CRC cells leads to a G1 arrest, and a switch in cellular programming from proliferation to differentiation (Van de Wetering et al., 2002). When β-catenin is deleted from the intestinal epithelium in mice, crypt structures are ablated and transit-amplifying cells

are lost, showing that Wnt/β-catenin signalling is essential for intestinal homeostasis (Fevr et al., 2007). Dickkopf1 (Dkk1) is a secreted Wnt inhibitor that binds the Wnt co-receptor, LRP5/6. The crucial role of Dkk1 was shown in the development of knockout mice, in which the deletion was embryonic lethal and led to cranial defects, highlighting the importance of Dkk1 in development. Conversely, overexpression of Dkk1 in the mouse intestine leads to the cessation of proliferation, complete with the absence of nuclear β-catenin in the crypts (Pinto, Gregorieff, Begthel, & Clevers, 2003). While a significant body of research exists showing the importance for Wnt signalling in the small intestine, Dkk1 antagonism of Wnt signalling has also been used to show that Wnt signalling is essential in colon proliferation as well as in the small intestine (Kuhnert et al., 2004). These data, plus the activation of the Wnt pathway in the initiation of colorectal cancer, indicates that Wnt signalling is the overarching factor that drives proliferation within intestinal crypts.

While the factors that drive intestinal homeostasis are well established, understanding how the canonical Wnt signalling pathway interacts with and regulates the intestinal stem cell pool has been the focus of recent efforts. Van der Flier et al, using gene-expression analysis, identified ~80 Wnt/TC4 target genes in the intestine. One of the genes most restricted in crypt expression is Lgr5 (Gpr49) (Van der Flier et al., 2007). $Lgr5^{+}$ stem cells reside within the crypt base of the intestine in a pattern like the SC zone model mentioned earlier. Supporting the theory that Lgr5+

cells comprise the SC pool of the intestinal epithelium, they have been shown to form self-organising proliferating crypt-villus units *in vitro* (Sato et al., 2009). Lineage tracing analysis using a LacZ reporter has shown that Lgr5+ cell progeny can generate all of the epithelial cell types (Barker et al., 2007). LacZ lineage tracing means that progeny from a progenitor cell can be tracked by observation of the 'blue ribbon' propagating up intestinal crypts. All of the differentiated epithelial cell types, such as enterocytes, goblet cells, enteroendocrine cells and Paneth cells can be found within the 'blue ribbons' produced by LacZ-Lgr5+ cells in the intestine. Moreover, these Lgr5+ lineages remain within the intestinal epithelium up to 14 months after reporter induction. These data suggest that Lgr5+ have long-term self-renewal and multipotency and are therefore bona fide adult stem cells.

It has been proposed that these *Lgr5*$^{+}$ stem cells represent one of the cancer SC pools present during colorectal cancer. Lineage tracing mouse models have shown that *Lgr5*$^{+}$ cells can function as the cells-of-origin for intestinal tumours, and that ablation of the gene led to tumour regression in murine xenograft models (Sato et al., 2009; Schepers et al., 2012; Shimokawa et al., 2017; Yanai et al., 2017). Interestingly, tumour regrowth in this model occurred through the re-emergence of *Lgr5*$^{+}$ cancer SCs, highlighting their importance in intestinal tumour development, but also demonstrating again the cellular plasticity within the intestinal epithelium,

where following stem cell ablation, other epithelial cells can dedifferentiate to become Lgr5-positive.

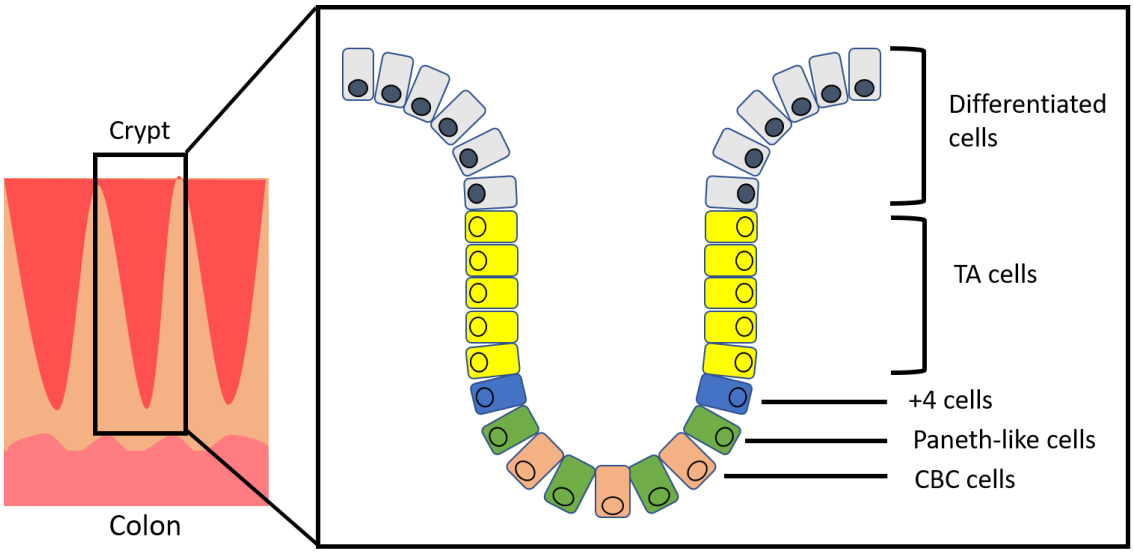


Figure 1.1 Structure of the crypt unit of the large intestine. Inset: The intestinal crypt-villus unit. The intestinal epithelial stem cells are thought to reside at the base of the crypt either as rapidly cycling CBC cells (brown), which are marked by Lgr5, or +4 cells (blue). Either SC pool may provide the stem cell population for the proliferating epithelium. CBCs are interdigitated between Paneth-like cells (green) which provide the epithelial niche for rapidly cycling SCs, are marked by Reg4. Transit Amplifying (TA) cells (yellow) migrate along crypt axis to form differentiated cells (white) in the epithelium: including Paneth-like cells, enteroendocrine cells, goblet cells, and enterocytes.

1.1.5 The genetic contribution to CRC

Genetics contribute to susceptibility to CRC, the progression of the disease and resistance of the disease to treatments. Therefore understanding the genetic basis of colorectal cancers can both help inform patients and help tailor prophylactic and reactive treatments (S. D. Markowitz & Bertagnolli, 2009).

Most often, mutations occur that increase the activity of the Wnt signalling, a group of signal transduction pathways essential to the proper functioning of multiple tissues, contributing to aberrant growth of the epithelial tissue. For example, mutations occur in key Wnt regulatory proteins APC, β-catenin, and AXIN2 (Wanguo Liu et al., 2000; Mori et al., 1992).

1.1.5.1 Mendelian CRC syndromes

Less than 5% of CRCs are caused by the inheritance of a single faulty gene (Lynch & de la Chapelle, 2003). These diseases are known as Mendelian CRC syndromes. Most of these diseases lead to the predisposition to colorectal adenomas. For example, FAP is caused by a mutation in the Wnt signalling pathway gene *APC*, while MAP is a

result of a mutation in the base-excision repair gene *MUTYH*. Lynch Syndrome, or HNPCC, can be as a result of mutations to DNA mismatch repair genes, such as *MSH2*, or *MLH1*. Other Mendelian CRC syndromes include Juvenile polyposis, which can be as a result of a mutation to BMP pathway gene *SMAD4* (Tomlinson, 2015).

1.1.5.2 Familial CRC

While less than 5% of the total burden of CRC is as a result of Mendelian syndromes, it has been suggested that around 30% of CRC is heritable (Lichtenstein et al., 2000). Therefore, there is a significant portion of heritable CRC burden that is not accounted for. It is thought that these non-syndromic familial CRCs are made up of a mixture of low risk susceptibility variants/SNPs with environmental factors (discussed previously) (Hahn et al., 2016).

1.1.5.3 Sporadic CRC

Many of the genes mutated in heritable CRC are often somatically inactivated in sporadic CRC (that is cancers that occur in people without a genetic predisposition towards or family history of cancer), so are of interest. Making up 70% of all CRC

cases, sporadic CRC occurrence is commonly thought of as a 'pathway' of genetic alterations, with an example of a traditional pathway involving mutation of *APC*/*KRAS*, followed by *TP53*, and finally *TGF*-β mutation (Yamagishi, Kuroda, Imai, & Hiraishi, 2016). However alternative pathways involving rarer sequences of alterations have also been postulated, such as the pathway resulting in sporadic serrated CRC, which may initiate with a mutation in *BRAF* (Pancione, Remo, & Colantuoni, 2012).

1.1.5.4 Inactivating mutations in tumour-suppressing genes & activating mutations in oncogenes

The most common initial gene mutated in both inherited and sporadic colon cancer is APC, and this gene's mutational inactivation represents the initiating event in colorectal cancer. Both alleles of APC are inactivated in colonic tumours through mutations which cause the loss of the full-length protein (Goss & Groden, 2000). This alteration has wide-ranging consequences in the colonic epithelial cell, affecting cell cycle progression, migration, differentiation, and apoptosis. In homeostasis, Wnt signalling, in which APC acts as an essential component mediates these pathways.

Wnt signalling results in β-catenin translocation to the nucleus resulting in cellular activation of Wnt transcriptional programming. APC holds β-catenin in complex, in the cytoplasm and targets it for degradation by the destruction complex, thereby inhibiting its nuclear localisation and blocking the activation of signalling programmes. Upon activation of the pathway by specific Wnt ligands (e.g. Wnt1), β-catenin is released from APC and the inhibition is removed.

Mutational inactivation of the gene that encodes APC leads to β-catenin being aberrantly translocated to the nucleus, and consequently, constitutive activation of the canonical Wnt signalling pathway. The consequence of hereditary APC-inactivating mutations is the development of Familial Adenomatous Polyposis (FAP), a CRC predisposition syndrome that occurs in humans and demonstrates a direct, causal link between canonical Wnt signalling levels and the subsequent development of CRC. FAP patients also develop colonic adenomas (numbering in the hundreds to thousands) at an early age and 95% of patients with FAP will be diagnosed with CRC by 50 years of age. FAP occurs with mutations in APC between codons 1250 and 1464, with mutations at codon 1309 particularly common (Bertario et al., 2003; Mori et al., 1992; Nagase et al., 1992). Attenuated FAP (AFAP) is an ameliorated form of FAP, which Burt and colleagues found a lifetime risk of 69% of CRC and a median polyp number of 25 (Burt et al., 2004). Patients with AFAP also develop polyp/CRC development at an older age compared to FAP counterparts. Mutations between

codons 1445 and 1578 of the APC gene are particularly associated with AFAP (Caspari et al., 1995).

As well as representing a significant risk factor in developing CRC in family groups, most sporadic colorectal adenomas and cancers contain somatic inactivation of APC (at 50% and 80% respectively) (Goss & Groden, 2000). Some CRC cell lines have mutations in β-catenin, rendering the protein resistant to degradation by the APC inhibiting complex and constitutively activating Wnt signalling (Morin, 1997). In serrated CRC, where *APC* is not normally found mutated but *BRAF* is, the ubiquitin ligases RNF43 and ZNRF3 (which negatively regulate canonical Wnt signalling) are found to be commonly mutated in 24% and 30% of *BRAF* mutant/MSI cancers, respectively (Bond et al., 2016). The consequence of RNF43 mutations is that the FzD receptor, which binds Wnt ligand cannot be ubiquitinated and therefore is no longer targeted for degradation following stimulation. Treatment with a porcupine inhibitor (which inhibits the palmitolaytion of Wnt ligand and therefore prevents their secretion) reduces growth of *RNF43/ZNRF3* mutant cells *in vitro* and therefore a potential therapeutic for serrated CRC, where RNF43/ZNRF3 is mutated. R-spondin family members are modulators of canonical Wnt signalling and are also implicated in CRC development. Seshagiri et al showed that *RSPO2* and *RSPO3* are together involved in recurrent gene fusions in 10% of colonic tumours. These events were mutually exclusive with *APC* mutations, suggesting that they may have a role in Wnt

hyperactivation and tumour initiation. It was then later shown that these fusion events are sufficient to initiate tumourigenesis *in vivo* (T. Han et al., 2017; Seshagiri et al., 2012). As this alteration makes the tumours entirely Wnt-dependent, treatment with the Wnt secretion inhibitor LGK974 resolved tumourigenesis entirely. This represents another therapeutic opportunity in treating CRC sub-types by using Wnt-modulating drugs.

The p53 signalling pathway also exerts tumour suppressive effects within the colon. P53 mediates cell-cycle arrest and also has a cell-death checkpoint, which are normally triggered by cellular stresses (S. D. Markowitz & Bertagnolli, 2009). Mutational inactivation of the gene TP53 (tumour protein p53) is a formative step in the development of CRC, often denoting the transition from adenoma into invasive carcinomas (S. J. Baker et al., 1990). Baker et al found that most tumours with inactive TP53 had a combination of the loss of one copy of chromosome 17p and a mutation in the remaining copy of p53. In this work they postulate that the mutation is the rate limiting step of p53 inactivation (S. J. Baker et al., 1990). Using conditional deletion of *APC* in the p53-deficient adult murine intestine, Reed et al showed that loss of p53 did not alter the *APC*-null phenotype (Reed et al., 2008). It is suggested that any potential changes in Wnt regulation carried out by p53 are overwhelmed by the constitutive Wnt activation brought about by loss of *APC*. Nakayama et al, using conditional mutant of p53 in loss-of-function *APC* mouse model, showed that the

mutation in p53 accelerated submucosal invasion. The mutation also conferred morphological changes in organoids created from the mice, with features associated with invasiveness. Allografts using these organoids displayed malignant histology, including increased mesenchymal marker expression and increased numbers of myofibroblasts (Nakayama et al., 2017).

Another key tumour suppressor pathway turned off in the development of CRC is Transforming Growth Factor-β (TGF-β) signalling. TGF-β is a pleiotropic cytokine which plays roles in immunosuppression, cellular differentiation and in maintaining homeostasis. A third of human colorectal cancer cell lines possess somatic mutations in the receptor TGFBR2 leading to inactivation (S. Markowitz et al., 1995). Grady et al found that this gene harboured inactivating mutations in 90% of MSI CRC. It was found that, like mutations of the p53 pathway, inactivation of TGFBR2 in MSI tumours tightly correlated with the progression from adenoma to carcinoma. They also estimated half of MSS cancers have either mutations in TGFBR2 or inactivating mutations or deletion of the downstream TGF-β components SMAD2 or SMAD3 (W M Grady et al., 1999; William M. Grady et al., 1998).

Oncogenic mutations of MAPK signalling components RAS and BRAF occur in 37% and 13% of CRCs, respectively (Bos et al., 1987; Nosho et al., 2008). One member

of the RAS gene family, KRAS, is mutated to cause constitutive GTPase activity that activates RAF, thereby driving the MAPK signalling cascade. This pathway regulates transcription of genes important for the cell cycle such as cyclin-dependent kinase 2 (CDK2) (Dogan et al., 2017). A member of the RAF family, BRAF, is commonly found in CRC to have oncogenic mutations which drive its own serine-threonine kinase activity which again causes aberrant MAPK signalling (Rajagopalan et al., 2002; Siena, Sartore-Bianchi, Di Nicolantonio, Balfour, & Bardelli, 2009).

1.1.5.5 Growth factor pathways

Growth factor pathways are often activated in CRC. Epidermal Growth Factor (EGF) is a key protein for intestinal epithelial homeostasis, stimulating cell growth, proliferation, and differentiation. It signals via activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) oncogenic pathways (Amado et al., 2008; Jhawer et al., 2008). Clinical data has shown that certain

colorectal cancers with oncogenic mutations in these pathways are resistant to anti-EGFR CRC therapy. For example, the monoclonal antibody panitumumab, which targets EGFR (EGF-Receptor), is shown to be effective in slowing progression of CRC in patients with WT KRAS, but in not patients with KRAS mutations (Amado et al., 2008). Therefore, KRAS mutational status screening can help improve efficacy of anti-EGF therapies in CRC.

A common early step involves aberrant activation of the prostaglandin signalling cascade (Cha & DuBois, 2007). It has been found that this signalling can be activated by upregulation of cyclooxygenase-2 (COX-2), seen in 67% of colorectal cancers, and by the loss of 15-prostaglandin dehydrogenase (15-PGDH) expression, seen in in 80% of colorectal cancers (Fink et al., 2014; Yan et al., 2004). The use of NSAIDs in clinical trials have been shown to prevent new adenoma formation via inhibition of COX-2. For example, aspirin has been shown to moderately reduce adenoma formation in a randomised trial with 1121 patients with recent history of adenomas (Baron et al., 2003). Celecoxib, a specific COX-2 inhibitor, was also found to reduce adenoma formation in a similar trial (Bertagnolli et al., 2006).

PI3K is a lipid kinase, which operates in pathways important in neoplasia such as motility, proliferation, and adhesion via PI3K/AKT signalling. Samuels et al found

that PI3KCA, encoding the catalytic subunit of PI3K, contains somatic mutations in 32% of colorectal cancers (Samuels, 2004). It was also found that PI3KCA mutations correlate with invasive cancers, suggesting that this type of signalling is important for CRC progression (Samuels, 2004). Similarly, PTEN, a negative regulator of PI3K/AKT signalling, was reported to be mutated in 19.5% of primary sporadic colorectal cancers, while the loss-of-heterozygosity was observed in 17% of cancers (Nassif et al., 2004).

Vascular endothelial growth factor (VEGF) is normally produced during periods of tissue growth or injury, promoting angiogenesis. Angiogenic pathways have recently been highlighted as having a role in colorectal cancer. For example, VEGF expression at the deepest invasive site of tumour is the most statistically significant prognostic indicator in advanced colorectal carcinoma (Ellis, 2007). A clinical study in 2004 used an anti-VEGF antibody in CRC therapy, resulting in a 5 month improvement in median survival over standard therapy (Hurwitz et al., 2004). More recently work in CRC cells has shown that VEGF signalling mediates migration and invasion (Bhattacharya et al., 2017)

1.1.5.6 Genomic Instability

Numerous oncogenic mutations can be acquired through the loss of genomic stability. The most common way this happens is through chromosomal instability (CIN), which can alter chromosomal copy number and structure. Wild-type copies of tumour-suppressor genes, such as APC and P53, can be lost in this way (S. Baker et al., 1989; Morin, 1997; Samowitz et al., 1999).

Barber et al systematically identified somatic mutations of potential CIN genes in colorectal cancers. It was found that most of mutations were in putative genes that regulate sister chromatid cohesion. The same group further demonstrated that the down regulation of these genes in human cells leads to CIN and defects in chromatid cohesion (Barber et al., 2008). This indicates that much of the CIN in colorectal cancer is due to mutations of genes that normally maintain stability of the chromosomes during replication.

Another form of genomic instability is found during defective DNA-repair. This occurs when genes which are normally responsible for repairing base-base mismatches are inactivated. In Lynch syndrome (HNPCC or hereditary nonpolyposis colorectal cancer), the heritable inactivation of these genes (e.g. hMLH1), leads to an autosomal dominant condition that predisposes to CRC. However, the inactivation of these genes can also be acquired non-heritably, through methylation-associated

silencing. Approximately 2-4% of all CRCs are as a result of Lynch syndrome, making it the most common inherited CRC syndrome (Hampel et al., 2008). Lifetime risk of colorectal cancer for those with Lynch syndrome is 50-80% (Stoffel et al., 2009). Patients with Lynch syndrome typically experience colonic adenomas and cancer at a younger age than patients with sporadic forms of these neoplasms. These cancers often occur more proximally in the colon than most others. A key characteristic of Lynch syndrome associated CRC is the elevated level of microsatellite instability (MSI-H), which is typical of cancers which have defective DNA mismatch repair (MMR) genes. Patients are also particularly susceptible to endometrial cancers, and to a lesser extent, cancers of the stomach and ovary (Lynch & de la Chapelle, 2003).

Mismatch-repair defects can be identified by the presence of microsatellite instability (S. D. Markowitz & Bertagnolli, 2009). A meta-analysis in 2015 found that MSI status was not predictive of survival in CRC patients undergoing conventional 5FU-based chemotherapy (Webber, Kauffman, O'Connor, & Goddard, 2015). In fact, CRC patients with MSI-H cancer have better survival rates than their microsatellite stable (MSS) counterparts (Ribic et al., 2003). Sporadic MSI-H cancers are seen much more commonly (approximately 12% of CRC) than heritable MSI-H cancers such as Lynch syndrome (3%) (Gatalica, Vranic, Xiu, Swensen, & Reddy, 2016).

1.2 Wnt Signalling

Wnt signalling is one of the major pathways in development and homeostasis. The importance of this signalling is stressed by the presence of many Wnt-related pathologies (for example, in idiopathic pulmonary fibrosis in the lung and in wound healing in the skin) (Cheon et al., 2002; Chilosi et al., 2003). Wnt signalling is essential for intestinal epithelial homeostasis and loss of it leads to loss of crypt structures and epithelial progenitors (Fevr et al., 2007). As well as this mutation of the downstream Wnt signalling regulator *APC* results in CRC initiation, highlighting the importance of Wnt in cancer development (Groden et al., 1991).

Wnt signalling can be broadly split into the Wnt/β-catenin dependent (or canonical) and the less-well defined β-catenin independent (or non-canonical) pathways (figure 1.2). Within the non-canonical pathway two further subtypes exist, the Wnt/Ca^{2+} pathway and the Planar Cell Polarity (PCP) pathway. All of the Wnt signalling pathways can be activated by members of the Wnt ligand family (Wallingford, Vogeli, & Harland, 2001). Wnt homologues are associated with the activation of specific pathways. For example, Wnt4, Wnt5a and Wnt11 are thought to have the ability to drive non-canonical signalling in different tissues (for example in bone or in adipose tissue), while Wnt1, Wnt3a and Wnt8 have been known to

activate the canonical β-catenin-dependent pathway (Catalán et al., 2014; Chang et al., 2007; Niehrs, 2012). However, increasingly, there are examples where ligands can activate both pathways depending on context, for example in osteoblastogenesis, where the typically non-canonical ligand Wnt5a enhances Wnt/β-catenin signalling (Okamoto et al., 2015). Suggesting that these delineations require some refinement.

It is well known that canonical Wnt signalling is important in regulating the proliferation and differentiation of the intestinal epithelium (Samowitz et al., 1999). As previously discussed, $Lgr5^+$ cells within the intestinal crypt represent the stem cell for the intestinal epithelium, capable of differentiating into all epithelial cell types. The epithelial SC niche is comprised of different cell types depending on the location in the intestine where, in the small bowel Paneth cells act as a stem cell niche, whereas in the colon $Reg4^+$ cells provide the stem cell niche.

Wnt signalling is a conserved pathway and is central for embryonic development, tissue homeostasis and disease (Biechele, Cox, & Rossant, 2011; Fevr et al., 2007; Groden et al., 1991) Wnts are secreted glycoproteins which are ligands to the Frizzled (Fzd) transmembrane receptor family, which are found on the surface of Wnt-responsive cells (Kikuchi, Yamamoto, Sato, & Matsumoto, 2011). In addition

to this, the Wnt co-receptors Low-density lipoprotein receptor-related protein-5 (LRP5) and -6 (LRP6) are important in mediating Wnt signals. LRP5 can bind to Axin, in response to Wnt ligand, in order to stabilise β-catenin, while LRP6 can initiate Wnt/β-catenin signalling in response to its phosphorylation (Mao et al., 2001; X. Zeng et al., 2007). Janda et al demonstrated that FZD-LRP6/LRP6 heterodimers can form and can elicit Wnt/β-catenin signalling, like Wnt agonist action, independent of ligand. Moreover, it was shown that this heterodimer exhibits Wnt activity *in vivo* in the liver in order to promote Wnt signalling and metabolic resonation in hepatocytes (Janda et al., 2017). However, it is unlikely that these dimers occur in vivo, but this study does demonstrate that activation of the canonical Wnt signalling pathway can be manipulated for regenerative medicine.

The Wnt ligand-receptor interaction is complex and following stimulation a number of signal transduction cascades can be activated. These are detailed below. One explanation of the high levels of pathway complexity is the numerous possible Wnt ligand, Fzd and co-receptor combinations that can be achieved. In addition to the canonical Fzd/Lrp interaction, Wnt is also known to act with non-canonical Fzd and a wide range of co-receptors including ROR1, ROR2, RYK and PTK7. ROR2, a Tyrosine-protein kinase transmembrane receptor, contains an extracellular cysteine-rich domain (CRD) which resembles the Fzd Wnt binding site, and has been show to act as a receptor for Wnt5a to activate non-canonical Wnt signalling (Oishi et al., 2003).

In a similar fashion to FZD/LRP interactions, PTK7 and ROR2 were shown to form heterodimers in mammalian cells, while WNT5A directly interacted with both proteins leading to Wnt/PCP activation of JNK and cellular migration (Martinez et al., 2015).

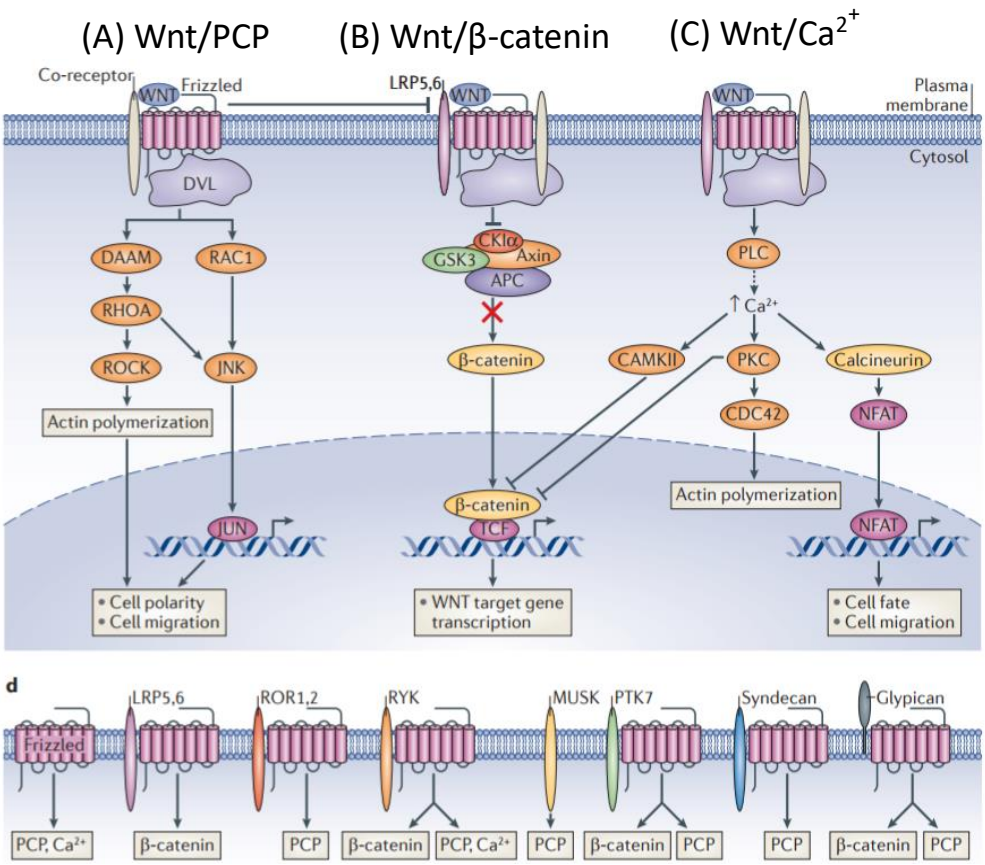


Figure 1.2 The Wnt signalling pathways. Schematic of the main Wnt pathways involving Wnt, Fzd and co-receptor interactions. **(A)** Wnt/PCP signalling. Non-canonical Wnt ligand forms a complex with Frizzled (Fzd) and a co-receptor. Fzd recruits Dvl to the receptor complex which activates downstream effects DAAM and RAC1. DAAM activates RhoA which enacts actin reorganisation through ROCK. RAC1 phosphorylates JNK which is an activator of Jun, part of the AP-1 transcription factor complex. This action can inhibit canonical Wnt signalling. **(B)** Wnt/β-catenin signalling. Canonical Wnt ligands induce a receptor complex involving Fzd, LRP5/6 and a co-receptor. Dvl is recruited to the complex which binds Axin and GSK3, thereby disrupting the β-catenin destruction complex, leaving β-catenin free to accumulate in the cytoplasm and translocate to the nucleus, where it interacts with TCF/LEF transcription co-factors to enact canonical Wnt target gene transcription. **(C)** Wnt/Ca^{2+} signalling. Non-canonical Wnt ligand forms a complex with Fzd and a co-receptor. Fzd recruits Dvl to the receptor complex leading to cytoplasmic Ca^{2+} accumulation. This results in transcriptional activation and cytoskeletal rearrangement. **(D)** Various Wnt receptor configurations and associated signalling outputs. Taken from Niehrs, 2012 (Niehrs, 2012).

1.2.1 Regulation of Wnt signalling

The Wnt signalling pathway is regulated at multiple points. In Wnt secretion, the ER-associated protein PORCN (Porcupine) palmitoylates Wnt ligands, which subsequently prevents accumulation of the ligand (Hausmann, Bänziger, & Basler, 2007; Takada et al., 2006) in the cell by promoting its transit though the golgi, via Wingless where it is secreted. Conditional deletion of PORCN in the skin of mouse embryos phenocopied β-catenin conditional mutants where both lacked hair follicles, suggesting that PORCN is essential for proper Wnt signalling (Wei Liu et al., 2012). PORCN inhibition has been used to block secretion of all Wnts in CRC-derived xenografts, leading to the inhibition of Wnt autocrine signalling and inhibiting the growth of colon cancers with RSPO translocations (Madan et al., 2016). Additionally, a phase I clinical study is currently underway in patients with solid malignancies using the PORCN inhibitor WNT974 (or LKG974) (Janku et al., 2015).

At the extracellular level, there are two classes of secreted Wnt antagonists. Secreted Frizzled-related proteins (sFRPs) were first identified in xenopus embryos. The have been found to bind directly to Wnts and act as a decoy receptor for the ligand, thereby inhibiting Wnt ligand from engaging the receptor complex (Salic, Kroll, Evans, & Kirschner, 1997). Deletion of (sFRP)-1 activates canonical Wnt signalling in

bone and increases trabecular bone formation in aged animals (Bodine et al., 2009) Another negative regulator that directly binds Wnt ligands is Wnt inhibitory factor 1 (WIF-1) (Malinauskas, Aricescu, Lu, Siebold, & Jones, 2011). The Dickkopf class of antagonists (such as Dkk1) inhibit Wnt signalling by binding the LRP5/6 components of the Wnt receptor complex. Deletion of a single allele of Dkk1 has been shown to lead to increased bone formation and bone mass, while the loss-of-heterozygosity of Dkk3 is associated with carcinogenesis yet reduced lymph node metastasis and better overall survival in head and neck squamous cell carcinoma (Katase et al., 2008), indicating that Dkk1 could have divergent and tissue specific roles. As LRP5/6 is recruited to the Wnt receptor complex for canonical Wnt signalling, theoretically only sFRPs can inhibit non-canonical Wnt signalling, and Dkk1 inhibitors have been restricted to the canonical pathway (Kawano, 2003) However, recent evidence indicates that Dkk1 might also function in the non-canonical pathway through regulating availability of non-canonical ligands (Caneparo et al., 2007; Tao, Liu, & Liu, 2013)

Wnt signalling can also be regulated through the composition of the Wnt receptor complex that is presented to the ligand. Frizzled is known to be present in the receptor complex in both canonical and non-canonical pathways. However, there are instances of Fzd-independent activation of the non-canonical pathway, whereby Wnt ligand can induce *VANGL2* phosphorylation through ROR2 (Wei Liu et al., 2012).

The previously mentioned LRP5/6 receptors are thought to be required in Wnt receptor complexes that can activate the canonical pathway (X. He, 2004). Interestingly, Bryja and colleagues found that the non-canonical Wnt ligand Wnt5a can interact with LRP6 but this does not activate the Wnt/β-catenin pathway. They also found that extracellular domains of LRP5/6 could inhibit the downstream target of Wnt5a, Rac1, suggesting that LRP5/6 can inhibit non-canonical Wnt signalling *in vivo,* and that this is achieved through the LRP5/6 receptor competing for Wnt ligands (Bryja et al., 2008). Another example of an intracellular regulator of the canonical Wnt pathway is DACT1 (Dishevelled Binding Antagonist Of Beta Catenin 1), which antagonises signalling through interaction with Dvl in hepatocellular carcinoma (Yau et al., 2005)

Known agonists for Wnt signalling include R-spondin and Norrin, both of which are thought to activate only Wnt/β-catenin signalling. Kazanskaya et al showed first that R-spondin2 could act synergistically with canonical Wnt ligands to activate the Wnt/β-catenin pathway. They also found R-spondins were often co-expressed with Wnts, and that R-spondin expression was dependant on Wnts, suggesting that R-spondin acts as a positive feedback mediator in canonical Wnt signalling (Kazanskaya et al., 2004). Using colorectal models, it was elucidated that RSPO functions as a ligand for the intestinal stem cell marker and orphan receptor LGR5 (Carmon, Gong, Lin, Thomas, & Liu, 2011). While this RSPO-LGR5 is important

for canonical control of intestinal homeostasis, it may be that similar mechanisms are in place in non-canonical signalling too. Later, it was shown that injection of human R-spondin1 into mice led to intestinal crypt proliferation, driven by activation of canonical Wnt signalling (K.-A. Kim, 2005). Norrin is an agonist for Fzd4, which combines to drive Wnt/β-catenin signalling in vascular development (Xu et al., 2004).

1.3 Wnt/β-catenin signalling

1.3.1 Wnt/β-catenin signalling in the absence of Wnt

β-catenin and its regulation in the cytosol represents the crux of canonical Wnt signalling (figure 1.3). The β-catenin destruction complex is well characterised, with the scaffolding protein Axin providing structural stability for the complex components GSK3, CK1α and β-catenin to come together. This complex allows the formation of the β-catenin destruction complex through sequential phosphorylation of β-catenin at serine 33, serine 37, serine 45 and threonine 41 performed by CK1α and GSK3 (Kimelman & Xu, 2006). Phosphorylation of the serine 33 and 37 residues creates a binding site for β-TrCP, an E3 ubiquitin ligase. The ubiquitination by β-TrCP results in β-catenin's degradation.

Axin also binds to another the scaffolding protein, APC, which itself binds to β-catenin. This complement ensures β-catenin is phosphorylated and subsequently degraded in the absence of Wnt (Stamos & Weis, 2013). Su et al showed that APC helps protect the β-TrCP binding sites in β-catenin from de-phosphorylation, thereby ensuring degradation of β-catenin (Su et al., 2008).

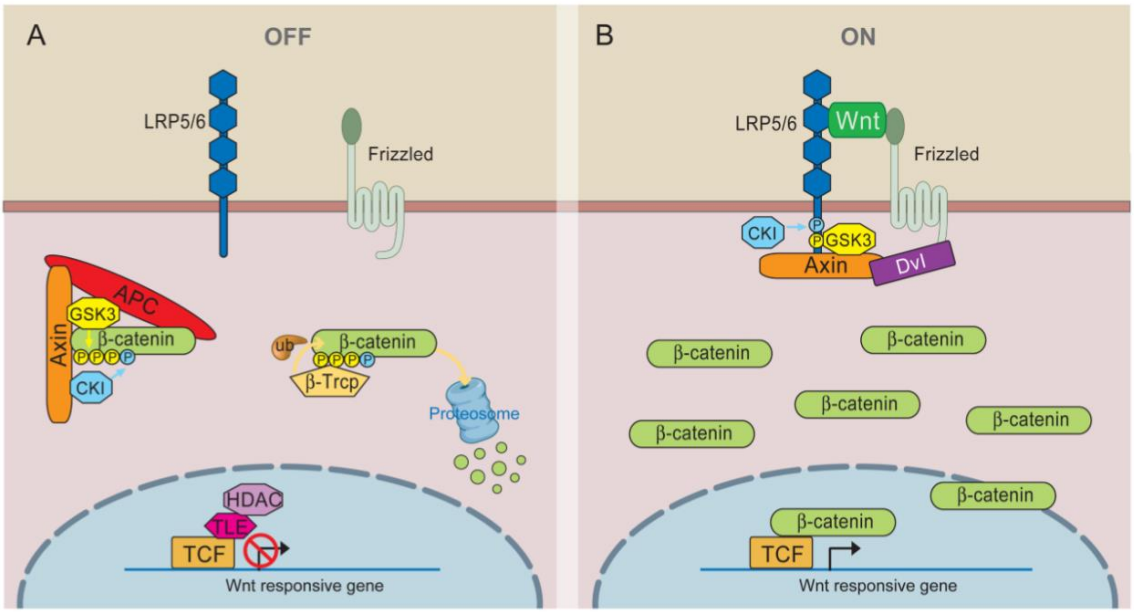


Figure 1.3 Canonical Wnt signalling in the absence and presence of Wnt

(a) In the absence of Wnt, cytoplasmic β-catenin is bound in a destruction complex formed by GSK3, APC, AXIN, and CK1. β-catenin is phosphorylated by CK1 and GSK3 which creates a binding site for β-TrCP, an E3 ubiquitin ligase. Subsequent ubiquitination targets β-catenin for degradationby the proteosome. (b) Wnt ligand forms a complex with the receptors LRP5/6 and Fzd. Fzd binds Dvl which leads to LRP5/6 phosphorylation through recruitment of Axin-GSK3-CK1 to the receptor complex. This disrupts the destruction complex-mediated phosphorylation and proteosomal degradation of β-catenin, allowing saturation in the cytoplasm. Subsequently β-catenin accumulates in the nucleus where it recruits TCF/LEF for activation of the Wnt transcriptional programme. Taken from Macdonald et al, 2009 (MacDonald, Tamai, & He, 2009).

1.3.2 Wnt/β-catenin (canonical) signalling in the presence of Wnt

The essential receptors Fzd and LRP5/6 are complexed in the presence of Wnt (figure 1.3). It has been shown that Wnt-induced phosphorylation of LRP5/6 is essential for Wnt/β-catenin signalling and the docking of the scaffolding protein Axin to the receptor complex at the membrane (Mao et al., 2001; Tamai et al., 2004). This key phosphorylation is carried by Axin-bound GSK3β and CK1α from the destruction complex (Xin Zeng et al., 2005).

Fzd is essential for this phosphorylation of LRP6 by Wnt ligands and it has been shown that Fzd interaction with Dvl is also required. Dishevelled (Dvl) is a cytoplasmic phosphoprotein that acts in the multiple Wnt signalling pathways directly downstream of Frizzled. Since it has been shown that Dvl interacts with Axin, it may be that recruitment of Axin-GSK3-CK1 for LRP6 phosphorylation is carried out by Fzd-Dvl (Bilic et al., 2007; Wallingford & Habas, 2005; X. Zeng et al., 2007), particularly as Dvl is known to form a polymer in the cytoplasmic side of the plasma membrane following Wnt ligand stimulation. It has been suggested that differential regulation of Dvl may represent the 'switch' between downstream canonical or non-canonical Wnt signalling (Simons et al., 2005). Grumaloto et al showed that the canonical Wnt ligand Wnt3a and non-canonical ligand Wnt5a specifically trigger unrelated co-receptors (LRP5/6 and ROR1/2, respectively). This triggering is achieved through a common mechanism of Wnt-Fzd interaction and recruitment of Dvl and GSK3. It was

also shown that GSK3 has the ability to phosphorylate both the canonical or non-canonical co-receptors (Grumolato et al., 2010). This shows that canonical and non-canonical Wnt compete at the cell surface for Fzd binding, and binding reciprocally inhibits the opposing pathway.

1.4 Non-canonical Wnt signalling

Non-canonical Wnt signalling, often referred to as β-Catenin-independent Wnt pathway, is classically divided into two branches: the Wnt/Planar Cell Polarity (Wnt/PCP) and the Wnt/Ca^{2+} pathways. Interactions between Wnt-Fzd (in a related way to canonical Wnt signalling) have been known to evoke non-canonical responses, as have Fzd independent interactions. These pathways diverge from the canonical signalling, as signalling does not directly control cytoplasmic β-catenin saturation to evoke transcriptional responses.

In 1982, Moon and colleagues first identified the ligand WNT5A in *Xenopus* when working to identify homologues of the Wnt ligand WNT1 (Christian, Gavin, McMahon, & Moon, 1991). Later, they found that injection of this ligand into *Xenopus* embryos led to developmental defects resulting from disruption of cellular movements, in contrast to WNT1 treatment which duplicated the embryonic axis (Moon, 1993). This data was the first suggestion that certain WNTs may act non-canonically. Since then, further Wnt ligands have been identified as initiating a non-canonical response. Jordan et al identified WNT4 as a key factor in female gonadal development, where the ligand attenuates recruitment of β-Catenin recruitment to steroidogenic factor 1 (SF1) binding sites (Jordan, Shen, Olaso, Ingraham, & Vilain,

2003). Heisenberg et al identified WNT11 as a requirement for proper convergent extension movements in zebrafish gastrulation (Tada & Smith, 2000). They consequently showed that WNT11 acts non-canonically as a dominant-negative Wnt11 mutant phenotype can be rescued by a truncated Dvl protein that fails to signal through the canonical pathway. These discoveries have led researchers to investigate components involved in non-canonical Wnt signalling other than ligands, and the identification of downstream proteins has helped define the Wnt/PCP and Wnt/Ca^{2+} pathways.

1.5 Planar Cell Polarity Signalling

Simple epithelial cells are polarised within tissue. This polarity is instrumental in the function of complex epithelial structures, by providing directionality to the epithelium. Planar Cell Polarity is a conserved pathway that establishes the anterior-posterior (also known as proximal-distal) axis of epithelial cells during morphogenesis. This is achieved through asymmetric localisation of the membrane-associated PCP components and intercellular communication to co-ordinate this localisation, and therefore polarity, between neighbouring cells.

1.5.1 Wnt/PCP signalling and the establishment of polarity in development

There is only a basic understanding of the PCP pathway, as opposed to the β-catenin dependent pathway, which has been studied extensively in several model species. It is known, however, that Wnt/PCP signalling plays a crucial role in tissue patterning in development.

PCP is established through the mutually exclusive localisation of core PCP proteins at opposing sides of the cell, which forms a pattern which is propagated across a plane of tissue. In Drosophila, Van-Gogh (Vang) forms a complex with Prickle and Fmi (mammalian homologue Celsr1) on the proximal side of the cell, whereas Fzd (mammalian homologues Fzd1-10), Dvl (Dvl1-3), Diego and Fmi form a complex on the distal side of the cell. In mammals, the *VANGL2* (Vang-like 2, homologue of Vang) complex prevents the Fzd complex from forming on the proximal side, whereas the Fzd complex prevents the *VANGL2* complex from forming on the distal side (Tree, Shulman, Scott, Gubb, & Axelrod, 2002). This effect propagates among cells in the same plane by interaction between proximal *VANGL2* and distal Fzd in adjacent cells (J. Wu & Mlodzik, 2008).

Proper polarisation of tissues by establishment of PCP is crucial in the development of many tissues. Convergent extension movements in *Xenopus* are controlled by Wnt11 which activates the non-canonical pathway via Dvl (Tada & Smith, 2000). Moreover, Wnt11 was also shown to mediate convergent extension movements in zebrafish (Heisenberg et al., 2000). Planar cell polarity was again implicated in the control of convergent extension by Lienkamp et al, who showed that PCP signalling is required for control over orientation during zebrafish kidney tubule elongation (Lienkamp et al., 2012). A lack of proper convergent extension in neuroepithelial development is one of the major reasons for neural tube defects

(NTDs). The homozygous loss-of-function (LOF) mutant *VANGL2* mouse, Looptail, *Lp* displays frequent NTDs as this has a mutation in the C-terminal domain of Vangl2 (S464N) which interferes with activation of downstream, non-canonical Wnt cascades. As well as this, additional PCP genes have been identified has being important in neural tube closure (Murdoch et al., 2014) and many phenocopy the Vangl2 Lp mutation.

PCP is also critical for proper orientation of cilia within cells. One of the most classical examples of PCP is within the mouse inner ear, where the asymmetric PCP patterning controls cilia orientation (Ehrenhofer-Murray, Rivier, & Rine, 1997). Multiciliated cells (MCCs) contain dozens of directionally beating cilia that act in concert to direct fluid flow across the tissue, and are crucial for development and homeostasis of the airway, the central nervous system, and the reproductive tracts. PCP signalling controls concerted cilia beating in all MCCs (Boutin et al., 2014; Guirao et al., 2010; Mitchell et al., 2009; T. J. Park, Mitchell, Abitua, Kintner, & Wallingford, 2008; Shi et al., 2014; Tissir et al., 2010; Vladar, Bayly, Sangoram, Scott, & Axelrod, 2012).

Other non-canonical Wnt pathway components have been implicated in a range of developmental and early life processes. Cervantes et al showed that Wnt5a

is essential for murine intestinal elongation in morphogenesis, and it was found cellular proliferation was reduced in this model, where Wnt5a is lost (Cervantes, Yamaguchi, & Hebrok, 2009). Further evidence of the importance of Wnt/PCP signalling in the intestine was found in 2015 when Jung colleagues identified the co-receptor PTK7 as a colonic SC marker in humans. Higher surface abundance of PTK7 correlated with self-renewal capacity, while a subset of $PTK7^{+}$ colonic SCs display label-retaining ability in differentiation towards enteroendocrine lineage (Jung et al., 2015).

While these core PCP components propagate polarity in this way between cells, they also play a crucial role in intracellular Wnt/PCP signalling. Upon activation of Fzd by a non-canonical Wnt ligand, Dishevelled (Dvl) is recruited to Fzd and co-receptor Ror2, which results in the activation of the GTPases RhoA, Rac1 and Cdc42 (figure 1.5) (Kirjavainen, Laos, Anttonen, & Pirvola, 2015; Niehrs, 2012). *VANGL2* plays an integral role here in regulating where the Fzd/Dvl/Ror2 complex forms. Several studies have shown that *VANGL2* can promote the internalisation of Fzd3, and this is done via *VANGL2* antagonism of Dvl1 phosphorylation of Fzd3 (M. Montcouquiol, 2006; Shafer, Onishi, Lo, Colakoglu, & Zou, 2011). This Fzd/Dvl/Ror2 complex promotes the polymerisation of actin and activation of the JNK-dependent transcription factors (e.g. AP-1, which includes cJun and cFos). More recently, this complex was implicated again in actin assembly where Gombos et al showed that

Wnt5a, Fzd, Dvl and *VANGL2* act in concert to activate the formin, dDAAM, a specific actin regulator (Gombos et al., 2015). These processes are thought to lead to changes in cellular migration, polarity, and gene expression.

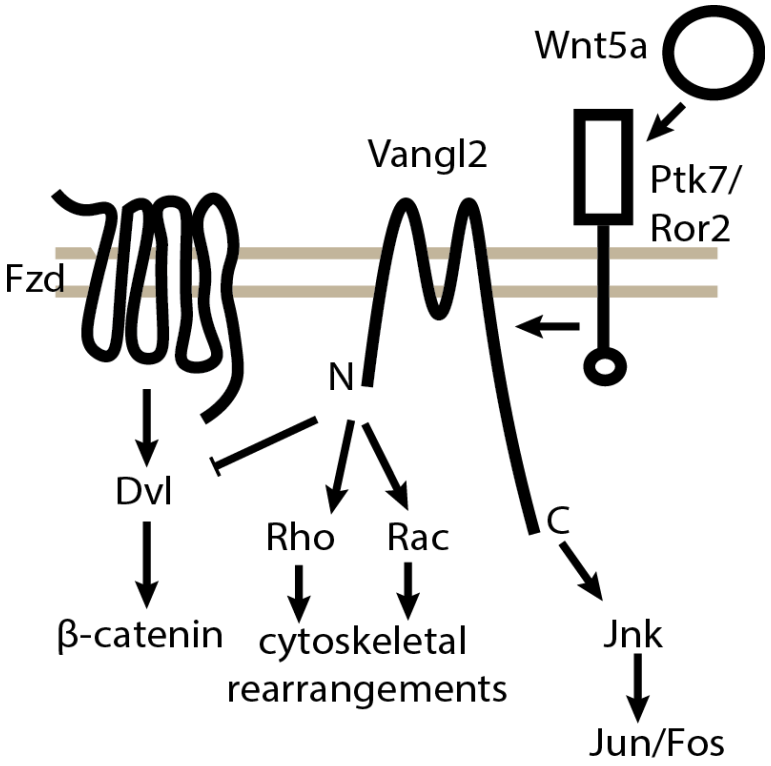


Figure 1.4 *VANGL2* in Wnt/PCP signalling

VANGL2 can form a non-canonical Wnt (e.g. Wnt5a) induced receptor complex with Ptk7/Ror2. The *VANGL2* cytoplasmic C-terminal PDZ binding domain can activate JNK signalling which leads to activation of the AP-1 transcription co-factors Jun/Fos. *VANGL2* cytoplasmic N-terminal tail contains Ser and Thr and are sites of phosphorylation and can activate Rho and Rac signalling which mediate cytoskeletal rearrangements. *VANGL2* can also act antagonistically towards the canonical Wnt signalling pathway via Dvl.

1.5.2 Wnt/PCP signalling in cancer

Significantly less is known about Wnt/PCP signalling within adult tissues. Most research looking at PCP in adult tissues has been centred on its dysregulation in cancers. In breast cancer *VANGL2* has been shown to interact with *VANGL1*, as well as the scaffold protein p62/SQSTM1. It was shown that the *VANGL2*-p62/SQSTM1-JNK signalling is upregulated in breast cancer patients with shorter survival (Puvirajesinghe et al., 2016).

There has been some recent work on the proteins downstream of *VANGL2* in cancer. For example, Prickle1 knockdown reduces metastasis of breast cancer cells, and is one of the many PCP components (including *VANGL2*) increased in CLL (Kaucká et al., 2013; Luga et al., 2012). Another downstream component, Scribble (Scrib1), has been shown to function as a tumour suppressor in liver cancer *in vivo* (Kapil et al., 2017). Scrib1 is required for the establishment of epithelial polarity and interacts with *VANGL2* via its PDZ-binding domain (Kallay, McNickle, Brennwald, Hubbard, & Braiterman, 2006). High expression of Scribble is correlated with worsened risk of relapse in breast cancer patients. However, other work has demonstrated that Scribble is an suppressor of tumour initiation in mice (Navarro et al., 2005; Zhan et al., 2008).

1.6 Wnt/Ca^{2+} Signalling

Another branch of the non-canonical Wnt pathway is Wnt/Ca^{2+} signalling. Like PCP signalling, it functions through Wnt/Fzd interaction and is crucial in development. It has been found that Wnt5a, Wnt11 and Fzd proteins are sufficient for intracellular Ca^{2+} release from endoplasmic reticulum (ER), while not affecting β-catenin stabilisation (Slusarski & Pelegri, 2007). In regions of the gastrulating embryo, 'waves' of Ca^{2+} are found suggesting it plays an essential role in pattern formation. Like the other Wnt pathways, this response is mediated by Fzd and Dsh proteins (Kühl, Sheldahl, Malbon, & Moon, 2000; Mudher et al., 2001; Sheldahl, Park, Malbon, & Moon, 1999). Ca^{2+} responsive proteins are subsequently activated by this signalling, including protein kinase C (PKC) and calcium/calmodulin-dependent kinase II (CAMKII). PKC can activate the small GTPase CDC42, a critical mediator of gastrulation. CAMKII has been shown to antagonise Wnt/β-catenin signalling via a pair of kinases (TAK1 and NLK) (Malinauskas et al., 2011; Sheldahl et al., 2003; Slusarski & Pelegri, 2007).

1.7 VANGL Planar Cell Polarity Protein 2 (*VANGL2*)

The Vangl, or Strabismus, protein family are Wnt/PCP pathway scaffolding proteins targeted to the cellular membrane. The two human members of the family, *VANGL1* and *VANGL2*, are membrane-spanning proteins with 73% amino-acid identity. *VANGL1* and *VANGL2* display genetic interaction in murine models, while *VANGL2* has been shown to have the ability to homodimerize with itself, as well as heterodimerise with *VANGL1* (Belotti et al., 2012; E. Torban et al., 2008).

1.7.1 Vangl structure

The Vangl proteins are the mammalian homologues of the *Drosophila melanogaster* protein Van Gogh (Vang/Strabismus). *VANGL2* and *VANGL1* possess four transmembrane domains and intracellular N- and C-termini, and are thought to interact with other proteins through their C-terminal coiled-coil domain or their PDZ-binding motif (figure 1.4) (Murdoch, Doudney, Paternotte, Copp, & Stanier, 2001). Their function is carried out entirely by protein-protein interactions as they lack inherent enzymatic activity. Human and murine *VANGL2* share 99% amino-acid identity. Belotti et al found evidence of endogenous *VANGL2-VANGL1* heterodimerisation in mice, of which N- and C- termini were not required (Belotti et

al., 2012). Furthermore, Torban et al found that *VANGL1* heterozygotes and *VANGL2* heterozygotes were viable and fertile, with subtle defects, while double *VANGL1* and *VANGL2* heterozygotes displayed severe developmental defects, including neural tube defects (NTDs), defects of the inner ear, and cardiac abnormality (E. Torban et al., 2008). These results show genetic interaction between the mammalian VANGL homologues, and a lack of redundancy between these genes. As *VANGL2* homozygotic loss-of-function (LOF) mutants display severe developmental defects, while *VANGL1* homozygotic LOF mutants do not, it has been proposed that *VANGL2* is dominant over *VANGL1*. It may be that the homodimerisation ability of *VANGL2* may be able to compensate for loss of *VANGL1* in development.

There is evidence that *VANGL2* acts in a signalling complex with Fzd to promote non-canonical Wnt signalling (Shafer et al., 2011; Treat et al., 2016). Dishevelled1 (Dvl1) can inhibit PCP signalling by hyper-phosphorylating Frizzled3 (Fzd3), whereas *VANGL2* antagonises the phosphorylation of Fzd3, causing its internalisation and thereby releasing inhibition of PCP signalling. Supporting this, Gao et al showed that Wnt5a mediates the phosphorylation of the *VANGL2* N-terminal cytoplasmic units in a *VANGL2*-ROR2 receptor complex, thereby controlling establishing PCP in chondrocytes (B. Gao et al., 2011).

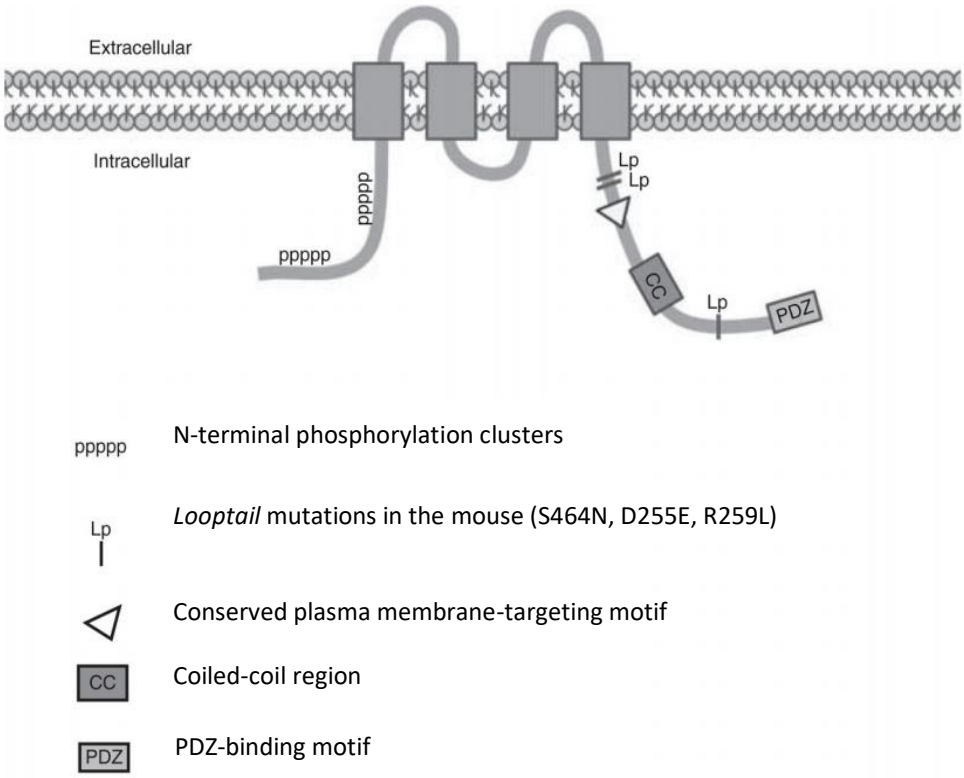


Figure 1.5 *VANGL2* structure

Vangls are membrane-associated proteins with four transmembrane domains. A cytoplasmic N-terminal tail possesses phosphorylation clusters at Ser and Thr residues. The cytoplasmic c-terminal tail contains a coiled-coil region and a PDZ binding-domain, both of which allow protein-protein interactions. The conserved membrane targeting motif allows transport from the trans-Golgi network. *Looptail* mutations on the c-terminal tail disrupt the membrane targeting ability of the protein. Taken from Hatakeyama et al, 2014 (Hatakeyama, Wald, Printsev, Ho, & Carraway, 2014).

1.7.2 *VANGL2* in development

In 1949, the *Looptail* (*Lp*) mouse was identified from inbreeding in a forward genetic screen (Strong & Hollander, 1949). Adult heterozygote (*Lp/+)* mice were characterised as having a variably kinked tail and unsteady or 'wobbly' head movements (Yin, Copley, Goodrich, & Deans, 2012). This is caused by disruption of the cilia within the cochlea, causing balance instability.

Having the correct PCP signalling in the epithelium is essential for the correct cell function and affects the morphology of that tissue. For example, in vertebrate development, a homozygous point mutation in the key signalling protein *VANGL2* causes a lack of convergent extension in the neuro-epithelium, leading to the failure of the neural tube to close (craniorachischisis, a severe neural tube defect) (Kit Doudney & Stanier, 2005). This is likely caused by a failure in growth cone guidance, shown by Shafer et al in the *Looptail* mouse (Shafer et al., 2011). In fact, human PCP genes are some of the most well-defined risk factors for NTDs (Wallingford, Niswander, Shaw, & Finnell, 2013). In patients with NTDs, mutations in many of the core PCP genes have been identified (Juriloff & Harris, 2012). Around 20% of patients with the NTD craniorachischisis contain putative mutations in *VANGL2*, Scrib, Dact1 (Dishevelled Binding Antagonist Of Beta Catenin 1), or Celsr1. In the heterozygote

form of the same mutation, the sensory hair cells in the mouse cochlea lose polarisation and have random orientation of cilia/stereocilia (Mireille Montcouquiol et al., 2003; Simons & Mlodzik, 2008). Mutations in other important non-canonical components Celsr and Scribble give the same phenotypes (Curtin et al., 2003; Mireille Montcouquiol et al., 2003). This literature indicates there is a global dependence on correct cellular orientation for epithelial tissues to function in the vertebrate.

Furthermore, embryonic lethal homozygotic (*Lp/Lp)* mutants were identified with cranioschisis, a neural tube defect. Kibar et al localised the *Lp* mouse mutation in *VANGL2*, and identified mutations within the gene which cause the Looptail phenotype (Kibar, Underhill, et al., 2001; Kibar, Vogan, et al., 2001). In addition to this, a study sequencing stillborn or mis-carried Han Chinese foetuses with severe NTDs found novel mutations within *VANGL2* (Y.-P. Lei et al., 2010). Identified mutations from this study were in similar positions to ones already shown to affect *VANGL2* function, and likely disrupt *VANGL2* interaction with Dvl (K. Doudney et al., 2005; Elena Torban, Wang, Groulx, & Gros, 2004). These mutations were exclusive to patients with NTDs and the lack of these mutations in healthy controls suggests that they have a lethal effect *in utero* which precludes their presence in living persons.

Mutations which cause the *Lp* phenotype are present within the C-terminal cytoplasmic domain, which contains a membrane targeting motif (Guo, Zanetti, & Schekman, 2013). It is was shown that the *Lp* mutation affects the membrane localisation of *VANGL2*, and results in its degradation by the proteasome (Iliescu, Gravel, Horth, Kibar, & Gros, 2011). However, another study suggested that the *Lp* mutation impaired the binding of *VANGL2* to Dvl proteins (Elena Torban et al., 2004). The same mutation in the lung also impairs organisation of the alveolar epithelium and causes a more constricted luminal space. These abnormalities are phenocopied by inhibition of Rho kinase, indicating that this Wnt/PCP factor downstream of *VANGL2* plays a role in lung epithelial organisation (Yates, Schnatwinkel, et al., 2010). There is also evidence that this mutation causes defects in the developing heart and in hair follicle patterning (Cetera, Leybova, Woo, Deans, & Devenport, 2017; Devenport & Fuchs, 2008; Phillips et al., 2008; Tatin et al., 2013). As well as this, *VANGL2* has been shown to be necessary for normal heart, lung and kidney development in vertebrates. In the cardiac outflow tract development of *Lp* mice, the extension of polarised membrane protrusions and the reorganisation of the actin cytoskeleton are inhibited in myocardial cells, indicating that this is a result of a defect in cellular polarity or migration (Deborah J. Henderson, Phillips, & Chaudhry, 2006). *VANGL2* has previously been shown to act via RhoA, which along with ROCK1 carries out cytoskeletal rearrangement and cellular movements, to regulate polarised cell

movements during the developing proximal outflow tract myocardium (Phillips, Murdoch, Chaudhry, Copp, & Henderson, 2005).

1.7.3 *VANGL2* has a developing role in cancer

As shown, *VANGL2* has been well characterised in mammalian development, but its effect in adult tissue homeostasis is still not known.

The tumour sample database The Cancer Genome Atlas (TCGA) found *VANGL2* was consistently upregulated or amplified in breast, ovarian and uterine carcinomas (Table 1). It has been shown that 24% of breast invasive carcinoma samples show upregulation *VANGL2* expression alongside 13% of samples containing *VANGL2* copy number amplification. *VANGL1* shows less strong trends in correlation with cancers, however it has been found in a separate analysis by Anastas et al to be a predictor in tumour recurrence in breast cancer patients (Hatakeyama et al., 2014).

This suggests that the Vangl proteins may be important regulators of cancer development in certain contexts. Kho et al, in 2009, showed that *VANGL1* is required for the invasiveness of CRC cells, and that *VANGL1* expression positively correlated

with human CRC stage. They also found that *VANGL1* promotes cell migration and invasion in colorectal cancer by forming a complex with Dvl and PKCδ to change cell motility (Kho et al., 2009). Similarly, Lee et al found that short-interfering RNA (siRNA)-mediated knockdown of *VANGL1* suppressed the progression and metastasis in colonic tumours in mice. It was found that *VANGL1* interacts with a PKC inhibitor (PKCI), which promotes invasiveness, and it was proposed that *VANGL1* interacts with PKCI to promote invasiveness/metastasis (Lee et al., 2005).

While we can find implications of *VANGL2*'s importance in cancer development using large-scale gene expression and copy number analyses, mechanistic insight into how it functions remains sparse. *VANGL2*, in recreating a similar role it plays in zebrafish gastrulation, inhibits cellular motility and cell surface expression of MMP2/MMP14 levels in fibrosarcoma cells. This implicates *VANGL2* in cell migration and extracellular matrix remodelling (Cantrell & Jessen, 2010; B. Blairanne Williams, Mundell, Dunlap, & Jessen, 2012).

PCP signalling may play a role in suppressing canonical Wnt signalling, which is the classical tumour-initiating stimulus. Mikels and Nusse found Wnt5a ligand can inhibit Wnt/β-catenin signalling, depending on the receptor context (Mikels & Nusse, 2006). This effect was mediated by the orphan tyrosine kinase ROR2, and did not

influence cellular calcium levels, suggesting that PCP signalling carries out this inhibition. Park and Moon found *VANGL2* has been shown to abrogate canonical Wnt signalling upstream of β-catenin via recruitment of Dvl1 to the plasma membrane, while promoting activation of the PCP pathways via phosphorylation of the c-Jun as part of the AP-1 transcriptional complex (which also includes Fos) (M. Park & Moon, 2002). Piazzi et al found that *VANGL2* promoter methylation is associated with higher tumour grade and BRAF mutation in human colon cancer. They also found that *VANGL2* overexpression inhibits Wnt/β-catenin signalling in CRC cell lines (Piazzi et al., 2013). This evidence suggests that *VANGL2* and PCP signalling functions as a tumour suppressor within the context of canonical Wnt-driven cancers such as CRC.

1.8 Hypothesis & Aims

I hypothesise that *VANGL2* acts to regulate mammalian intestinal homeostasis and colorectal cancer.

The aims of the thesis are as follows:

1. Determine if *VANGL2* is expressed within the healthy adult intestine and in colorectal cancers.
2. Evaluate whether alteration of *VANGL2* expression plays a role in human colorectal cancer pathology.
3. Establish the role *VANGL2* plays in mammalian intestinal homeostasis and in cancer, using murine LOF and intestinal cancer models.
4. Determine the mechanisms through which *VANGL2* is acting in the intestinal epithelium and establish how signalling through *VANGL2* regulates intestinal phenotypes.

Chapter 2: Materials & Methods

2.1 Buffers and Solutions

2.1.1 Methacarn Fixative

60% Methanol

30% Chloroform

10% Glacial Acetic Acid

2.1.2 TE antigen retrieval Buffer (1 L)

Tris 1.21 g

EDTA 0.37 g

Distilled water 1 L

Mix to dissolve. Adjust pH to 9.0 then add 0.5 mL of Tween 20 (Sigma).

2.1.3 Sodium Citrate antigen retrieval Buffer (1 L)

Tri-sodium citrate (dihydrate) 2.94 g

Distilled water 1 L

Mix to dissolve. Adjust pH to 6.0 then add 0.5 mL of Tween 20 (Sigma).

2.1.4 3% Hydrogen Peroxide (10 mL)

Hydrogen Peroxide 30% solution (Sigma) 1 mL

Distilled water 9 mL

2.1.5 Trypsin-Versene Solution

0.025% Trypsin and 0.01% Versene (EDTA) solution in PBS

2.1.6 Flow Cytometry Buffer

PBS

0.05% Sodium Azide (Sigma)

0.5% Bovine Serum Albumin (Sigma)

2.2 Mouse models

Mice were maintained and bred at University of Edinburgh facilities according to UK Home Office guidelines. Wild type C57BL/6 mice were bred in-house. Mice were maintained in 12h light-dark cycles and had access to food and water ad libitum.

2.2.1 VillinCre^{ERT} VANGL2$^{flox/flox}$ mice

VillinCre^{ERT} mice, which contain an inducible *Cre* recombinase gene under control of the *Villin1* promoter, were crossed with *VANGL2$^{flox/flox}$* mice, which have *loxP* recombination target sites flanking exon 4 (the transmembrane component) of the gene (Madison et al., 2002). *VANGL2^{flox}* mice were provided by Professor Deborah Henderson (Newcastle University) (Ramsbottom et al., 2014). Mice were culled 5 days after induction were tamoxifen.

To assess long-term loss of *VANGL2* and tumourigenesis, mice were induced and aged for 6 months. Survival and tumour number was quantified as shown in section 2.2.3.

2.2.2 Villin-Cre^{ERT} VANGL2$^{flox/flox}$ Rosa$^{mT/mG}$ mice

VillinCre^{ERT} VANGL2$^{flox/flox}$ mice were crossed with *Rosa$^{mT/mG}$* mice (Jackson Laboratory) (Muzumdar, Tasic, Miyamichi, Li, & Luo, 2007). tdTomato (RFP) expression is found in all tissues, while *Cre*-expression tissue (in this case in *Villin*-expressing cells) undergo recombination resulting in EGFP expression. Mice were culled 5 days after induction with tamoxifen.

2.2.3 VillinCre^{ERT} VANGL2$^{flox/flox}$ APC$^{flox/WT}$ mice

VillinCre^{ERT} VANGL2$^{flox/flox}$ mice were crossed with *APC$^{flox/WT}$* animals. These mice had *loxP* recombination target sites flanking exon 14 in one allele of the *APC* gene (Colnot et al., 2004). Symptom surveillance was carried out over the duration of the experiment specifically noting hunching, rectal bleeding, anaemia, and weight loss. Mice were sacrificed when disease severity reached endpoint status (up to 300 days following induction).

For tumour quantification, mice intestines were flushed with PBS and opened longitudinally. Intestines were pinned and submerged in methacarn to allow detection of all intestinal polyps. Number of tumours was counted in the small intestine and in the colon. Diameter of all tumours was also measured. Cumulative tumour burden was calculated as the sum of all tumour diameters within each mouse. Average tumour diameter was calculated as the sum of all diameters divided by number of polyps.

2.2.4 Tamoxifen induction

Cre expression was induced with intravenous injections of tamoxifen (reconstituted in Ethanol, then diluted in corn oil at 40°C, both Sigma) at 80 mg/kg of bodyweight on two consecutive days.

2.3 Epithelial Isolation

The dissected mouse colon was isolated and washed with cold PBS. The colon was opened longitudinally and rinsed again with cold PBS before tissue was cut into small (2-3 mm) pieces. Tissue was washed with PBS before incubation with agitation in 25 mM EDTA in PBS at 4^0C for 30 minutes. EDTA solution was removed and tissue washed with PBS, using a motorized pipette, displacing the tissue 10 times. Tissue was allowed to settle and supernatant taken as one epithelial fraction. This process was repeated until four fractions were collected.

2.3.1 RNA extraction of epithelial cells

Following this, all fractions were combined and RNA extraction protocol was followed (Section 2.6.2).

2.3.2 Flow cytometry analysis of epithelial cells

Alternatively, for flow cytometry analysis of *Villin-Cre^{ERT} VANGL2$^{flox/flox}$ Rosa$^{mT/mG}$* mice, epithelial cells were digested into single cells using TrypLE Express (Gibco) for

15 minutes at 37^0C. Cells were re-suspended in flow cytometry buffer. Cells were analysed via flow cytometry for GFP (excitation peak 488 nm) and tdTomato (554 nm) expression (after gating for single cells). Samples were acquired on a FACSJazz benchtop flow cytometer (BDBiosciences). All flow cytometry data was analysed using FlowJo software (BDBiosciences). The %GFP+ cells between *VANGL2*$^{WT/WT}$ and *VANGL2*$^{flox/flox}$ was calculated.

2.4 Human tissue

2.4.1 Human CRC tissue sections

Slide sections comprised of colorectal cancer (CRC) and non-cancer human tissue were obtained from the Division of Pathology Laboratories at the Western General Hospital (tissue approval number: TGU-LAB-587).

2.4.2 Tissue microarray (TMA)

Pantomics TMAs COC1261 and COC1262 used, comprising 42 CRC patient cores paired with normal colonic tissue (www.pantomics.com).

2.5 Human CRC Database

Colorectal Adenocarcinoma (TCGA, Provisional) study used. Patient overall survival and disease-free survival data segregated into high-expression and unaltered-expression of *VANGL2* was downloaded from the Colorectal Adenocarcinoma (TCGA Research Network, Provisional) dataset through cBioPortal (www.cbioportal.org) on 10.08.17 (J. Gao et al., 2013). High and unaltered patient survival times were plotted,

median survival times were calculated. Differences in survival was tested for statistical significance using a Log-rank test ($p < 0.05$).

2.6 Molecular biology techniques

2.6.1 RNA Isolation & Quantification

A small piece of tissue (small intestinal or colonic whole tissue; or extracted colonic epithelial cells) was placed in 1 mL of TRIzol reagent (Invitrogen). Tissue was first homogenized using a steel bead and a TissueLyser LT machine (Qiagen) for 4 minutes at 50 Hz, with an additional 1 minute at 50 Hz as required. Samples were then centrifuged at 10,000 *g* for 3 minutes at 4°C to remove tissue debris and supernatants decanted into fresh eppendorf tubes.

Chloroform was added (200 µl per 1 mL TRIzol), samples inverted 10 times and incubated at room temperature (RT) for 3 minutes. Samples were centrifuged at 12,000 *g* for 10 minutes at 4^0C. The clear aqueous phase (containing total RNA) was removed and transferred to a new 1.5 mL eppendorf tube (carefully avoiding the white-coloured interphase containing genomic DNA, and the pink-coloured organic phase containing cellular debris). Isopropanol (500 µl per 1 mL TRIzol) was added,

samples inverted 10 times and left for 10 minutes at RT. Samples were then purified using the RNeasy Mini Kit (Qiagen). Purifications were performed according to manufacturer's instructions, with RNA eluted in a final volume of 30 µl of RNase-free water (Qiagen). RNA concentration and purity was analysed on a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). UV absorption was measured at 230, 260, and 280 nm. 1.5 µl of each RNA sample was used for measurements, with 1.5 µl of RNase-free water used to blank the machine. RNA measurement was calculated using absorbance at 260 nm. Optimally, RNA samples had an A_{260}:A_{280} ratio of > 1.8 (lower indicating DNA contamination) and an A_{260}:A_{230} ratio of > 1.4 (lower indicating phenol contamination)

2.6.2 Synthesis of cDNA and gene expression quantification via qRT-PCR

A fixed amount of 1 µg of RNA was used for the reverse transcription synthesis of cDNA from cellular RNA. Reverse transcription reactions were performed using QuantiTect Reverse Transcription Kit (Qiagen) using manufacturer's instructions.

SYBR Green I-based real-time PCR assays were performed on the LightCycler 480 instrument within 384-Well Reaction Plates (all Roche). Primers (QuantiTect Primer Assays, Qiagen) were purchased from Qiagen's Primer Assay library

(www.qiagen.com) and are shown in Table 2.6. Reactions were performed using SYBR Green System (Roche) according to manufacturer's instructions. cDNA samples were diluted 1:5 with nuclease-free water (Qiagen), and 5 µl of diluted cDNA was used per sample in a total reaction volume of 12.5 µl. Cycle conditions: 5 min pre-denaturation at 95°C, followed by 45 cycles of 10 s denaturation at 95°C, 20 s annealing at 60°C, and 20 s elongation at 72°C. All samples were performed in technical triplicate. A no-transcriptase control was used to assess DNA contamination and nuclease-free water was used as a non-template control. *PPIA* (Peptidylprolyl Isomerase A) was used as housekeeping gene for normalisation in all qRT-PCR analyses. This was chosen on recommendation from Dr Kevin Myant (CRUK, Edinburgh) as the optimal intestinal transcript to normalise against (with low variability of expression within the intestine).

Table 2.6 Quantitative PCR primer assays

Gene	Entrez Gene ID	QuantiTect Primer Assay
AXIN2	12006	Mm_Axin2_1_SG
BMP4	12159	Mm_Bmp4_1_SG
β-CATENIN	12443	Mm_Ccnd1_1_SG
CMYC	17869	Mm_Myc_1_SG
CTGF	14219	Mm_Ctgf_1_SG
CYR61	16007	Mm_Cyr61_1_SG
DCN	13179	Mm_Dcn_1_SG
DVL1	13542	Mm_Dvl1_1_SG
DVL2	13543	Mm_Dvl2_1_SG
DVL3	13544	Mm_Dvl3_1_SG
FOS1	14281	Mm_Fos_1_SG
IGFBP4	16010	Mm_Igfbp4_1_SG
KRT19	16669	Mm_Krt19_1_SG
LEF1	16842	Mm_Lef1_1_SG
LGR5	14160	Mm_Gpr49_1_SG
MMP2	17390	Mm_Mmp2_1_SG

MMP7	17393	Mm_Mmp7_1_SG
MMP9	17395	Mm_Mmp9_1_SG
PKCϑ	18761	Mm_Prkcq_1_SG
PPIA	268373	Mm_Ppia_1_SG
PRICKLE1	106042	Mm_Prickle1_1_SG
PRICKLE2	243548	Mm_Prickle2_2_SG
PRICKLE3	54630	Mm_Prickle3_1_SG
PRICKLE4	381104	Mm_Prickle4_2_SG
ROR2	26564	Mm_Ror2_1_SG
SCRIB1	105782	Mm_Scrib_1_SG
SOX9	20682	Mm_Sox9_1_SG
SPARC	20692	Mm_Sparc_1_SG
TIMP1	21857	Mm_Timp1_1_SG
TIMP3	21859	Mm_Timp3_1_SG
VANGL1	229658	Mm_Vangl1_1_SG
VANGL2	93840	Mm_Vangl2_1_SG
WNT4	22417	Mm_Wnt4_1_SG
WNT5A	22418	Mm_Wnt5a_1_SG
WNT5B	22419	Mm_Wnt5b_1_SG

WNT11	22411	Mm_Wnt11_1_SG
αSMA	11475	Mm_Acta2_1_SG

2.6.3 RIN scores, RNAseq, and Transcriptome pathway analysis

RNA quality assessment for RNA sequencing was performed by Agnes Gallacher (Medical Research Centre Human Genetics Unit (MRC HGU), University of Edinburgh) using the Agilent 2100 Bioanalyser. An RNA integrity number (RIN) of ≥7 was deemed appropriate for sequencing.

RNA samples were provided to GATC Biotech for Illumina RNA sequencing (www.gatc-biotech.com). Dr. Stuart Aitken of the MRC HGU performed quality control and mapping of the source data, followed by calculation of FPKM (fragments per kilobase of exon per million reads mapped) for differential expression analysis between genotypes, and finally identification of differentially expressed genes and transcripts ($q < 0.05$).

Overexpression analysis on ConsensusPathDB (CPDB) was performed on the gene list of significantly upregulated genes and on the list of significantly downregulated genes in the *VANGL2*$^{Flox/Flox}$ tissue compared to control. Genes are mapped to pathway-based gene sets (only pathways with two or more matched genes included). Pathway sources used include Reactome, KEGG, PID, Wikipathways, and NetPath. Significantly

(q < 0.05) over-represented pathways are selected for and top 10 from each gene list shown in Table 4.2.5B (Kamburov et al., 2011) (http://cpdb.molgen.mpg.de/CPDB).

2.6.4 RNA ISH (RNAScope)

RNA in situ hybridization (was performed by Aquila Histoplex using RNAScope probes (ACDBio) against *VANGL2*, *VANGL1*, WNT5A, PTK7, PPIB (positive control), and a non-targeting negative control (nt-control) (www.histoplex.co.uk). Staining was visualised using Diaminobenzidine (DAB) chromogen.

2.7 Histology & Immunohistochemistry

2.7.1 Intestinal histology

Mice were sacrificed and intestine removed. Dissected intestinal tissue (small intestine or colon) was washed with PBS, opened longitudinally before being rolled into a 'swiss-roll' as previously published (Moolenbeek & Ruitenberg, 1981). Tissue was fixed in methacarn for 24 hours then washed in Methanol for 24 hours (alternatively for immunofluorescent staining, tissue was fixed in 4% paraformaldehyde, PFA, solution for 24 hours). Tissue was then washed in fresh methanol for 1 hour, followed by incubation in ethanol, xylene, and paraffin (3 x 30 minutes each). All steps for tissue processing performed in Tissue-Tek VIP Infiltration Processor (Sakura). Processed tissue was embedded in paraffin and tissue sections cut at 4 µm thickness. Tissue sections were baked in an oven overnight at 50°C. Sections were deparaffinized in 3 x 5 min washes of xylene followed by rehydration in a graduated ethanol series.

For histological evaluation of *Rosa*$^{mT/mG}$ mice, colons were dissected and flash frozen in O.C.T. compound (Tissue-Tek) using a 2-methylbutane/dry ice bath. Sections were cut at 10 µm thickness. Cryosections were fixed in a 1:1 acetone:methanol mixture before mounting with with Vectashield containing DAPI (Vector Laboratories).

2.7.2 H&E

Deparaffinised sections were stained in Harris haematoxylin for 5 minutes and washed in running tap water for 5 minutes. Staining was differentiated in 1% acid alcohol for 30 seconds before washing again for 1 minute. Developing the stain was performed in saturated lithium carbonate solution for 30 seconds followed by washing in tap water for 5 minutes. Tissue was counterstained in eosin for 1 minute and tissue dehydrated in ethanol before clearing tissue in xylene and mounting slides with DPX mounting medium (Sigma).

Tumour histopathology assessment was carried out by Dr. Tim Kendall (MRC Centre for Inflammation Research, University of Edinburgh) using H&E-stained sections from induced *VillinCre^{ERT} VANGL2$^{flox/flox}$ APC$^{flox/WT}$* and *VillinCre^{ERT} VANGL2$^{WT/WT}$ APC$^{flox/WT}$* mice.

2.7.3 IHC: Chromogenic staining

Antigen retrieval (if required) was performed using pre-warmed TE or Sodium Citrate buffer. Slides treated with 3% Hydrogen Peroxide for 15 minutes at RT to block

endogenous peroxidase. Slides blocked with Avidin and Biotin block solution each for 15 minutes at RT. Protein block (Spring Bioscience) was added for 30 minutes at RT. Primary antibodies (Table 2.7) in antibody diluent reagent solution (Invitrogen) were incubated overnight at 4°C. Tissue incubated in -biotinylated secondary antibody at RT for 30 minutes, followed by treatment with VECTASTAIN Elite ABC-HRP (Vector Laboratories) treatment for 30 minutes at RT. Slides are incubated in DAB substrate (Abcam, made according to manufacturer's instructions) for 5 minutes at RT. Slides counterstained with haematoxylin, dehydrated in alcohol, and washed in xylene before mounting with DPX mounting medium (Sigma). PBS washes used in between all antibody incubation steps.

2.7.4 IHC: Immunofluorescent staining

Antigen retrieval (if needed) was performed using pre-warmed TE or Sodium Citrate buffer. Retrieval was performed in a conventional microwave oven for the time indicated in table 2.7. Protein block (0.5% BSA, 0.5% casein in PBS, Spring Bioscience) was added for 30 minutes at RT. Primary antibodies (Table 2.7) in antibody diluent reagent solution (Invitrogen) were incubated overnight at 4°C and washed in PBS 3

times for 5 minutes each. Tissue incubated in fluorescently conjugated secondary antibody in the dark at RT for 30 minutes and subsequently washed in PBS 3 times for 5 minutes each. Slides were mounted with Vectashield containing DAPI (Vector Laboratories).

2.7.5 Definiens IHC Quantification

Quantitative IHC analyses were performed using Definiens Tissue Studio (Definiens, Carlsbad, CA) image analysis software. Whole-scans of intestinal sections were imported for analysis. User-guided machine learning is employed to generate an analysis that defines positive areas of expression. Regions of interest (ROIs, for example epithelium or adenomas) were identified for analysis using manually drawn areas. Nuclei are detected from haematoxylin counter-staining, while DAB chromogenic staining is recognized as positive areas of expression. Images were then batch processed to define and quantify areas of expression along with intensity of expression. Proliferative stains were quantified as a percentage of positive nuclei, while all other stains were quantified using a quickscore (QS). QS = average intensity of expression x % area of expression. Three or more sections were quantified per genotype.

2.7.6 *VillinCre^{ERT} Rosa$^{mT/mG}$* crypt recombination counting

Sections were prepared as described in section 2.7.1 from colons from two induced *VillinCre^{ERT} Rosa$^{mT/mG}$* mice. Ten fields-of-view (FOV) from each colon was captured using a confocal microscope at 10x magnification. Total number of crypts and number of GFP^{+} crypts were counted and %GFP^{+} was calculated.

Table 2.7 List of antibodies for immunohistochemistry (IHC)

Specificity	Host	Dilution	Source	Antigen Retrieval (PFA-fixed tissue)
VANGL2	Rabbit	1:100	Sigma/Atlas	10 min in TE buffer
WNT5A	Rabbit	1:100	LS Bio	10 min in TE buffer
PTK7	Rabbit	1:100	Proteintech	10 min in TE buffer
KI67	Rabbit	1:2000	Abcam	
PCNA (PC10)	Mouse	1:4000	Cell Signalling	
DCN	Goat	1:50	R&D Systems	
SPARC	Goat	1:100	R&D Systems	
COL1	Goat	1:1000	SouthernBiotech	
LAMININ	Rabbit	1:500	Abcam	
CTGF	Rabbit	1:500	Abcam	
C-JUN (60A8)	Rabbit	1:300	Cell Signalling	
PHOSPHO-C-JUN (SER73)	Rabbit	1:100	Cell Signalling	
Specificity	**Host**	**Dilution**	**Source**	
Anti-Rabbit Biotinylated	Goat	1:500	Vector Labs	
Anti-Mouse Biotinylated	Goat	1:500	Vector Labs	
Anti-Goat Biotinylated	Rabbit	1:500	Vector Labs	
Specificity	**Host**	**Dilution**	**Source**	
Anti-Rabbit Alexa Fluor 488	Donkey	1:500	Life Technologies	
Anti-Rabbit Alexa Fluor 594	Donkey	1:500	Life Technologies	

2.8 Intestinal and Colonic Organoids

2.8.1 Colonic organoid culture

The dissected murine colon was flushed with cold PBS and cut longitudinally to expose the lumen. Exposed lumen was washed again with PBS and cut into 1-2 mm pieces. Tissue was washed 3-4 times with cold PBS before incubation in 25 mM EDTA in PBS for 30 minutes with agitation at 4°C. After EDTA aspiration, tissue was washed with 10 mL of cold PBS and vigorous pipetting. The supernatant (containing crypts) was collected as one fraction. This PBS washing was repeated until four fractions were collected, and each fraction was analysed under a light microscope for crypt enrichment. Fractions most enriched for crypts were combined and filtered through a 70 µM cell filter (Fisher Scientific) and washed with ADF media. Crypts were then centrifuged at 100G to remove single epithelial cells. Crypts were then spun at 300G and the resultant pellet resuspended in Matrigel (Corning). 500 µl of Matrigel was used per colon used for culture, and 20 µl was plated per well in a 24-well tissue culture plate. ADF-based organoid growth media was added, featuring EGF, Noggin, and R-Spondin1 growth factors. Organoids were incubated at 37°C, 5% CO_2 and culture media renewed every 2-3 days.

2.8.2 Colonic adenoma organoid culture

Dissected murine colonic adenoma was rinsed with PBS before being cut into 1 mm pieces. Adenoma tissue was then incubated with agitation in 25 mM EDTA in PBS for 15 minutes at 4°C. Tissue was then incubated with Trypsin-Versene solution for 15 minutes at 37°C. Tissue was washed by centrifugation at 300 *g* for 5 minutes and passed through a 70 µM cell filter (Fisher Scientific) and washed with ADF media. Cells are spun down at 300 *g* and pellet re-suspended with Matrigel (Corning), with around 500 µL Matrigel used per 24-well tissue culture plate. ADF-based organoid growth media was added without recombinant R-Spondin1 or Wnt3a ligand to select for neoplastic epithelial cells (featuring Wnt hyper-activation). Organoids were incubated at 37°C, 5% CO_2 and culture media renewed every 2-3 days.

Complete ADF for organoid culture:

Advanced DMEM/F12 medium (Gibco)

100 U/ml penicillin & 100 µg/ml Streptomycin (MRC HGU)

2 mM L-glutamine (MRC HGU)

10mM HEPES Buffer

1x N2 Supplement (Thermo Fisher Scientific)

1x B27 Supplement (Thermo Fisher Scientific)

50 ng/mL EGF (Invitrogen)

100 ng/mL Noggin (Peprotech)

500 ng/mL R-Spondin1 (R&D) *

100 ng/mL Wnt3a (Peprotech) *

*not used for adenoma organoid culture

2.8.3 RNA extraction

For organoid RNA extraction, TRIzol (Invitrogen) was added directly to Matrigel droplets following removal of culture medium. RNA was subsequently extracted from the organoids using the RNA extraction protocol (Section 2.4.2).

2.9 Statistical analysis

All statistical analyses were performed using Graphpad Prism 7 (Graphpad Software Inc.). Unless otherwise indicated in figure legends, data analysis was performed as follows;

For comparisons of two groups, a parametric student's t-test, unpaired, and two-tailed was used. When three or more groups were analysed by one-way ANOVA, with a Tukey's multiple comparisons post-test. *** = $P < 0.001$, ** = $P < 0.01$, * $P < 0.05$, N.S. (not significant) $P > 0.05$.

Chapter 3: Characterisation of VANGL2 & non-canonical Wnt Signalling components in the intestinal epithelium

3.1 Introduction

Loss-of function (LOF) mutations in *VANGL2* have been identified in humans with neural tube defects (NTDs), however it has been difficult to define whether these mutations are causative, as they have generally been identified using targeted sequencing. Therefore, there could be other causative variants that interact with mutations in *VANGL2* or are able to cause NTDs independent of *VANGL2*. To address the limitations of site directed sequencing, the core Wnt/Planar Cell Polarity (PCP) gene, *VANGL2,* has long been studied for its role in vertebrate development using mice (Kibar, Vogan, et al., 2001; van Abeelen & Raven, 1968). Mice used include knock-outs using *VANGL2* floxed alleles and point mutations in *VANGL2* such as the LOF *Looptail* model, which has a *VANGL2*^{S464N} point mutation (D. J. Henderson et al., 2001; Y.-P. Lei et al., 2010; E. Torban et al., 2008). While similar phenotypes are found in mice and patients with *VANGL1* mutations, homozygous mutants do not display the severe developmental conditions compared to the *VANGL2*, suggesting *VANGL2* plays the predominant role in regulating development. While *VANGL2* has shown the ability to homodimerize, *VANGL1* can only form heterodimers with *VANGL2* (Belotti

et al., 2012; E. Torban et al., 2008). It may be that the ability for *VANGL2* to bind itself may compensate for any loss of functional *VANGL1* in development.

While it has been shown that *VANGL2* is expressed in the stomach epithelium in development and is required for proper fore-stomach morphogenesis, the role of *VANGL2* in intestinal development has yet to be fully elucidated (Satoh, Matsuyama, Takemura, Aizawa, & Shimono, 2008; Elena Torban et al., 2007). It has been reported, however, that *VANGL2* is expressed in the intestinal tube during organogenesis from E9.5 and increases over time, while Wnt/PCP ligand WNT5A is essential for the elongation and development of the small intestine in mice (Cervantes et al., 2009). Given the compelling evidence for *VANGL2*'s critical role in epithelial morphogenesis, it must be considered that Vangl2 may have a role in the homeostasis and/or disease of epithelial tissues, such as the intestinal epithelium.

To date, there has been no research carried out examining the role of *VANGL2* in intestinal epithelium. Given this and given *VANGL2* has previously been shown to inhibit canonical Wnt signalling, an essential driver of intestinal epithelial renewal, researching of *VANGL2* and related non-canonical signalling genes would be beneficial (Hayes, Naito, Daulat, Angers, & Ciruna, 2013) to define the role of Vangl2 in intestinal epithelial homeostasis.

VANGL2 has been implicated in several cancers, including in breast cancer where transcriptional upregulation and genomic amplification of the gene leads to the promotion of epithelial proliferation through JNK signalling, and ultimately poorer prognosis in patients (Puvirajesinghe et al., 2016). However, there is little analysis of the role of *VANGL2* in colorectal cancer. Piazzi et al found that *VANGL2* is frequently silenced by methylation in microsatellite instable (MSI) colorectal cancer cell lines (Piazzi et al., 2013). It was also shown that methylated *VANGL2* associated with CRCs that are MSI, higher grade, BRAF mutant and have proximal colon location. Moreover, in the colorectal cancer cell line SW480 overexpressing *VANGL2*, it was found that β-catenin levels were decreased concurrently with proliferation, indicating that *VANGL2* expression may be able to inhibit canonical Wnt signalling. Similarly, fellow Wnt/PCP signalling components WNT5A and ROR2 are also frequently repressed due to promoter hyper-methylation, and overexpression of these genes resulted in tumour suppressive behaviour *in vitro* (Lara et al., 2010; Ying et al., 2008). These data are in agreement with previous data showing *VANGL2* acts as a suppressor of invasion in fibrosarcoma cells (Cantrell & Jessen, 2010). Here cells with suppressed *VANGL2* expression have augmented migratory ability, and have increased invasiveness through improved matrix metalloproteinase (MMP) activity. Therefore, *VANGL2* may play a role in regulating human CRC, and it is of interest to explore further.

3.2 Results

3.2.1 Quantification and localisation *VANGL2* transcript within the murine intestine

Given that there is little definitive information about *VANGL2* expression in the colon, I wanted to determine the presence or absence of *VANGL2* within the murine intestine, and to define the expression of *VANGL2* longitudinally along the intestine. As well as this, investigating the expression pattern of other PCP components in the intestine was also of interest, as *VANGL2* functions in tandem with several other proteins to orchestrate the PCP signal. The murine intestine serves well as a model of human intestinal homeostasis, with similar gross architecture, epithelial structure, and cell types, as well as similar pathways governing the epithelium (as evidenced by the conserved Wnt signalling pathway). Therefore, the mouse intestine used here for characterising of the expression of *VANGL2*. Adult C57BL/6J mice had the full length of their intestines (comprising the small intestine starting from the duodenum, the caecum, and the colon up to the anal canal) harvested. Intestines were divided into 3cm pieces, RNA extracted and cDNA synthesised. Each 3cm piece of tissue was assigned to each of the intestinal segments (duodenum, jejunum, ileum, caecum, and colon) and qRT-PCR analysis was carried out looking at PCP component expression (Figure 3.2.1A).

I found that *VANGL2* transcript is detectable in all sections of the whole mouse intestine (using a cut-off Ct of 40 for detection), along with several other genes which are components of the PCP signalling pathway (Figure 3.2.1A). This includes the *VANGL2* homologue *VANGL1* (shown to be dysregulated in some human cancers), the Dishevelled (Dvl) gene family (crucial for PCP signalling during development), and the Prickle family (a key PCP regulatory factor). The Wnt family ligands shown to activate the non-canonical pathway (including WNT5A shown to be a key prognostic marker in other epithelial cancer), ROR2 (known to interact with WNT5A to activate PCP signalling) and Scrib1 (established to be mutated in the neural tube defect spina bifida) are also included (Andre, Song, Kim, Kispert, & Yang, 2015; Etheridge et al., 2008; Gibbs et al., 2016; Hatakeyama et al., 2014; Heinonen, Vanegas, Lew, Krosl, & Perreault, 2011; Jenny, Reynolds-Kenneally, Das, Burnett, & Mlodzik, 2005; Y. Lei et al., 2013; Martinez et al., 2015; Saling et al., 2017; Shafer et al., 2011; Wang, 2006). Expression levels, relative to those of the duodenum, vary across the intestine for each gene.

While I have shown that there is transcript expression of these PCP components in the colon, this analysis includes transcripts from the whole intestine and not just epithelium. Given the prominent role PCP and *VANGL2* play within epithelial tissue organisation, I narrowed my investigation to epithelial tissue. To analyse component expression specifically within the colonic epithelium, and to

localise individual transcripts, I performed RNA in situ hybridisation (ISH). Mouse colonic tissue was probed for *VANGL2*, *VANGL1*, *WNT5A*, or *PTK7* mRNA in a chromogenic assay that is sensitive enough to detect single transcripts (Figure 3.2.1B). Using this method of defining mRNA positivity I found that transcripts of each of these genes were found in the murine colonic epithelium, validating the qRT-PCR data by showing these are expressed and localising expression to the epithelium. Transcripts of each of these genes were found broadly along the crypt axis. The presence of *VANGL2* and its homologue *VANGL1*, the non-canonical ligand WNT5A, and the Wnt/PCP signalling co-receptor PTK7 support further investigation into the function of *VANGL2* signalling in the intestine.

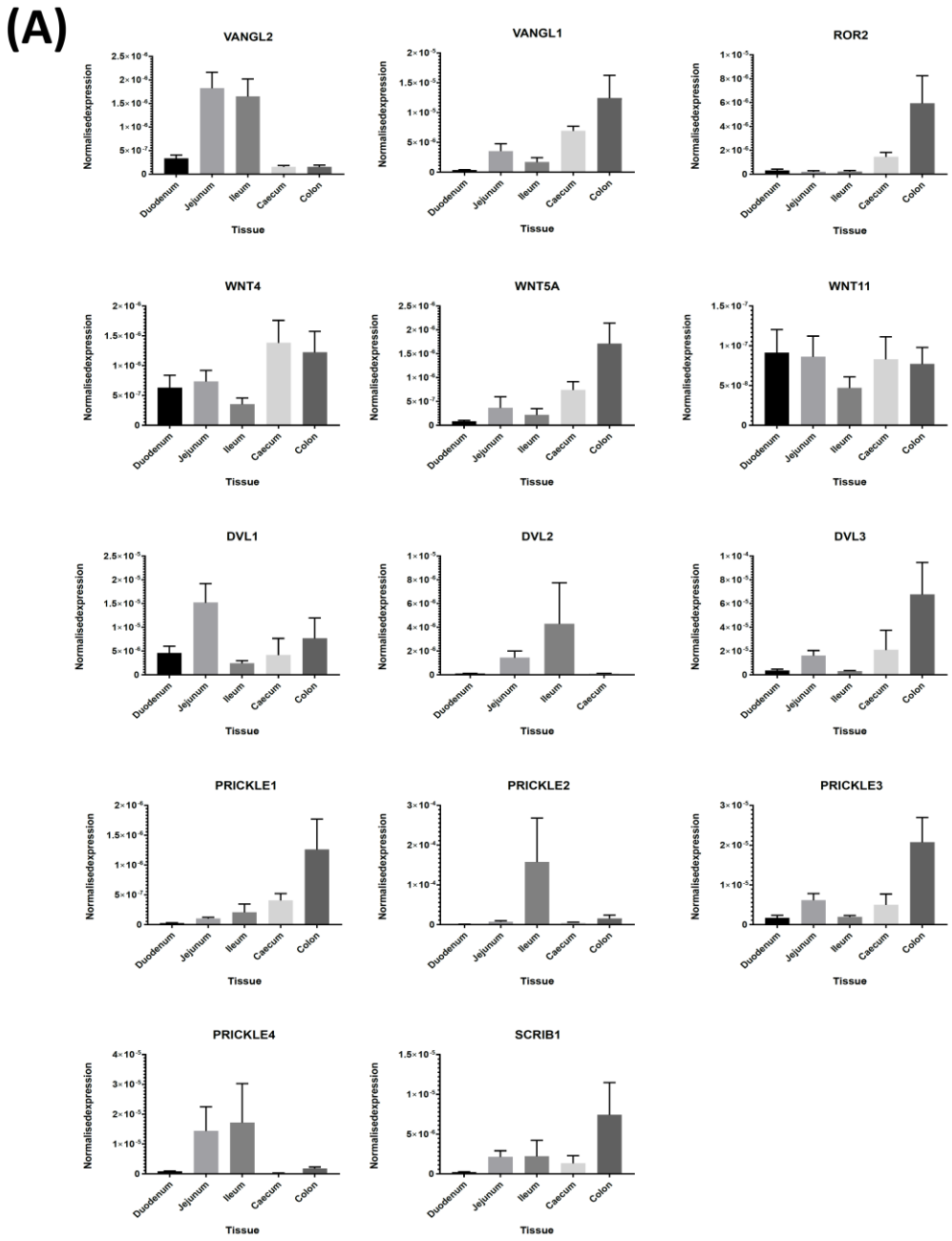


Figure 3.2.1 Quantification and localisation of planar cell polarity gene (PCP) transcripts within the mouse intestine

(A) qRT-PCR analysing whole-intestinal RNA expression of PCP genes through the duodenum, jejunum, ileum, caecum, and colon of the mouse intestine. RNA was extracted from segments of mouse intestine and cDNA synthesised. qRT-PCR was then performed targeting the indicated genes. All expression normalised to Ppia. Mean (SD) values shown. *n* = 4.

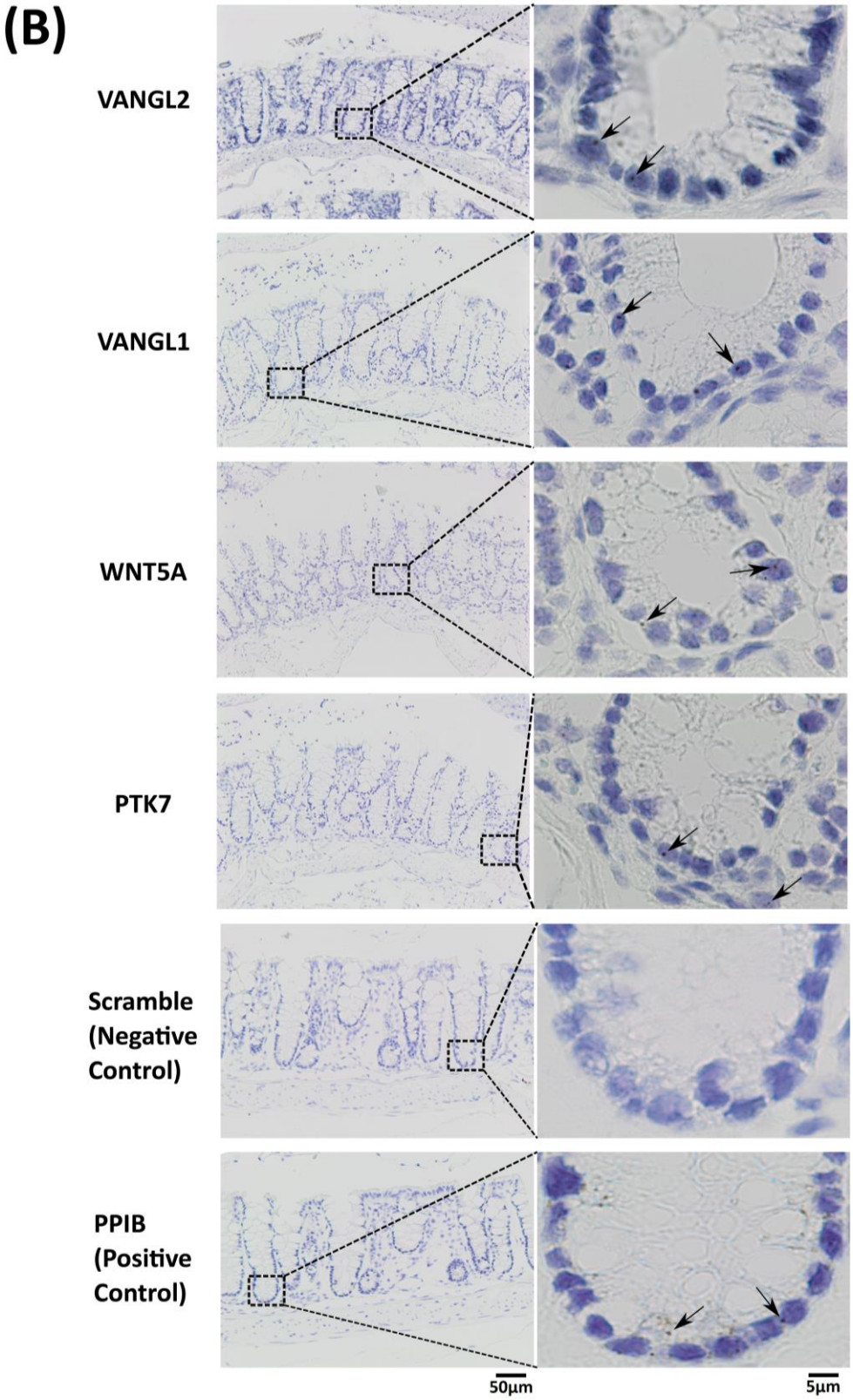


Figure 3.2.1 Quantification and localisation of planar cell polarity gene (PCP) transcripts within the mouse intestine - *continued*

(B) RNA ISH analysis of PCP components in fixed mouse colon. ISH performed localising transcripts using probes targeting VANGL2, VANGL1, WNT5A, and PTK7. Negative (scramble) control and positive (PPIB) control probes also used. Representative images used from 3 stains. Brown chromogen-stained areas are indicative of a transcript (Arrows). 10x and 100x magnifications used.

3.2.2 Localisation of *VANGL2* protein within the murine colon

With the pattern of *VANGL2* transcription within the colon defined, I proceeded to investigate *VANGL2* expression at the protein level. This would define the presence and localisation of *VANGL2* protein within the mouse colonic epithelium. Fixed mouse colon was stained using an anti-*VANGL2* antibody, and I visualised *VANGL2* protein in the murine colon using DAB (Figure 3.2.2A). *VANGL2* was found to be expressed broadly across the crypt axis (similar to what I have defined with *VANGL2* transcripts using ISH), however, the protein was found to generally have cytoplasmic or membranous cellular localisation. It is thought that *VANGL2* needs to be localised to the membrane for activity in PCP signalling (S. Li et al., 2011). I also assessed the Wnt/PCP ligand WNT5A for expression in the colon (Figure 3.2.2A). It has previously been shown that this protein functions in PCP signalling in mice (Qian et al., 2007). WNT5A is localised at the base of the colonic crypts and surrounding stromal cells. Given the presence of the core PCP proteins *VANGL2* and WNT5A (one of the Wnt ligands that conventionally activate PCP signalling), I investigated if both proteins can be found in the same location, which could suggest interaction (direct or otherwise. I conducted immunofluorescent stains on both proteins and found that they co-localised in the murine colonic epithelium (Figure 3.2.2B). Another transmembrane protein, PTK7, has been shown to promote PCP signalling and has been shown to interact genetically with *VANGL2* (Hayes et al., 2013; Lu et al., 2004). PTK7 has also

been identified as a colonic SC marker with lineage tracing ability (Jung et al., 2015). Immunofluorescence analysis in the mouse colon shows co-localisation of both *VANGL2* and *PTK7* within the base of the colonic crypts (Figure 3.2.2C). Interestingly, this co-localisation was not seen within the small intestine (Figure 3.2.2D). My data illustrates the presence of a Wnt/PCP 'signalling hub' within the murine colon, suggesting there is Wnt/PCP signalling in the adult intestine.

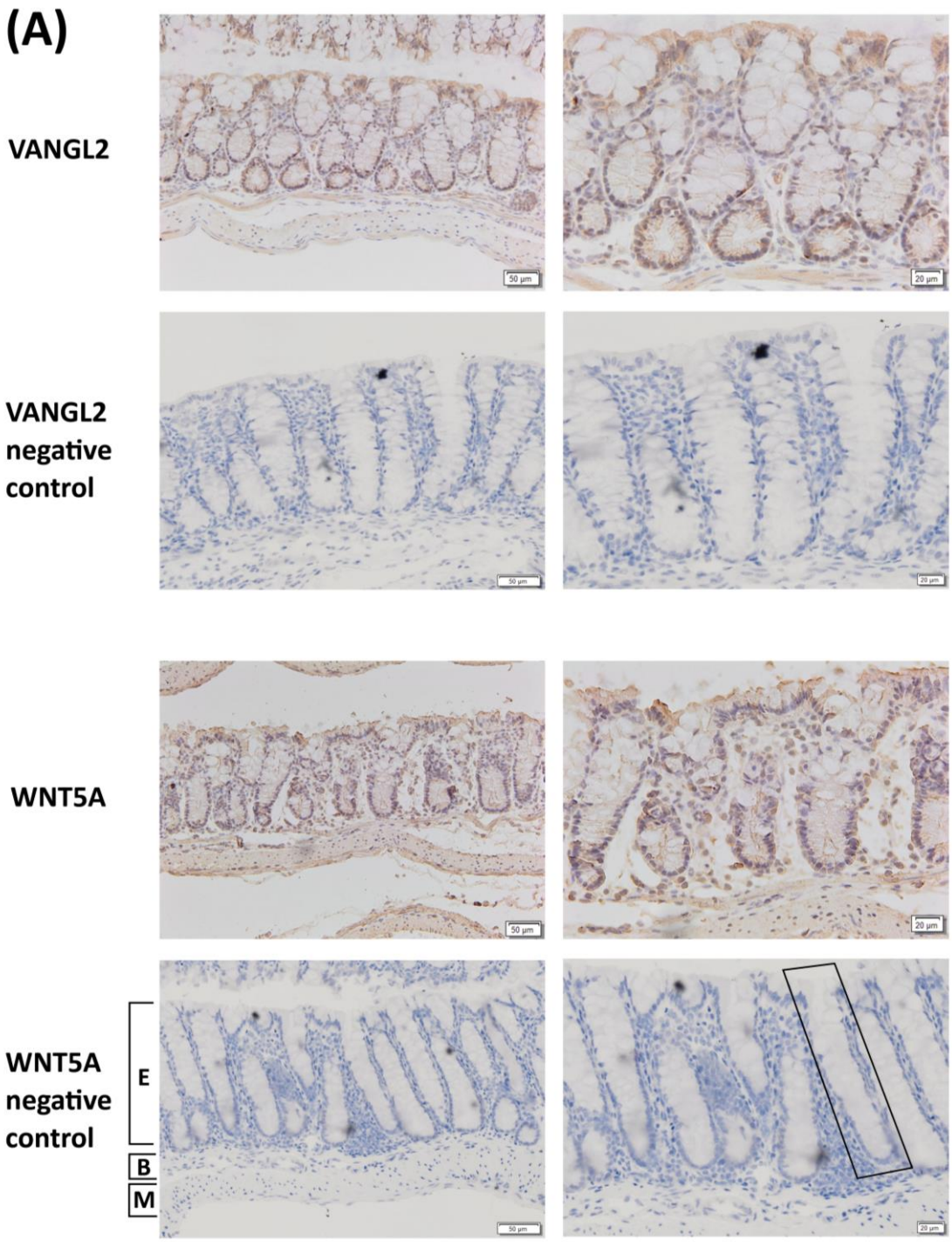


Figure 3.2.2 Localisation of VANGL2 protein within the murine colon

(A) VANGL2 and WNT5A staining of the wild-type (WT) mouse colon. Note the broad range of VANGL2 expression along the crypt axis, and the largely cytoplasmic/membraneous localisation. Note the localisation of WNT5A to the base of the crypts and surrounding stromal cells. Negative controls are tissue stained minus primary antibody. Representative images of 5 seperate whole-scanned colons. The final image set features annotation for the structure of the colon. On the left, E = Epithelium, B = Basement membrane, M = Lamina propria. On the right, a single colonic crypt is identified.

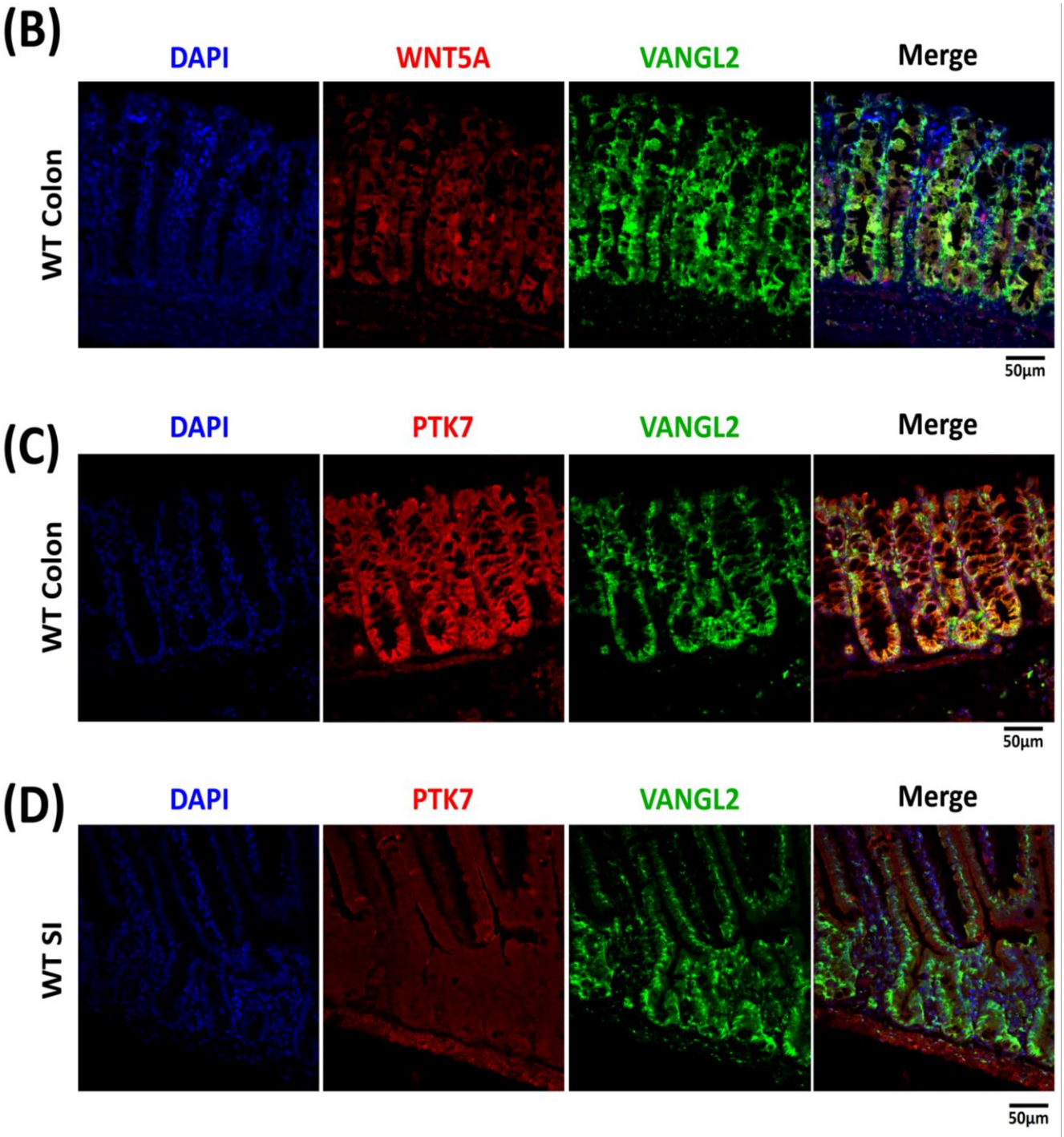


Figure 3.2.2 Localisation of VANGL2 protein within the murine colon - *continued*

(B) Immunofluorescent co-stain for VANGL2 and WNT5A was performed in the WT mouse colon and staining observed by confocal microscopy. **(C)** Immunofluorescent co-stain for VANGL2 and PTK7 was performed in the WT mouse colon, and **(D)** in the WT mouse small intestine (SI). Representative images used from 3 stained sections.

3.2.3 Localisation of *VANGL2* in the human colon

Given that *VANGL2* is expressed at the transcript and protein level in the murine colonic epithelium, I then sought to establish if *VANGL2* is present in an analogous manner in the human colonic epithelium, to validate the murine colon as a suitable model for looking at the role of *VANGL2* in human colorectal disease. Fixed human non-cancerous colonic tissue was stained using the same *VANGL2* antibody that we used in the murine stains (Figure 3.2.3A). Representing the first characterisation of *VANGL2* in the human colon, I found that *VANGL2* protein is localised to the colonic epithelium and is broadly expressed along the crypt axis. Recapitulating part of the PCP 'signalling hub' seen in the mouse. *VANGL2* and PTK7 were also seen to be co-localised within the human colon (Figure 3.2.3B). More specifically, PTK7 staining was restricted to the crypt base, which is important given the reports on PTK7 acting as a colonic stem cell marker (Jung et al., 2015). This supports the data seen in the mouse and I therefore decided to characterise the expression of *VANGL2* in human CRC.

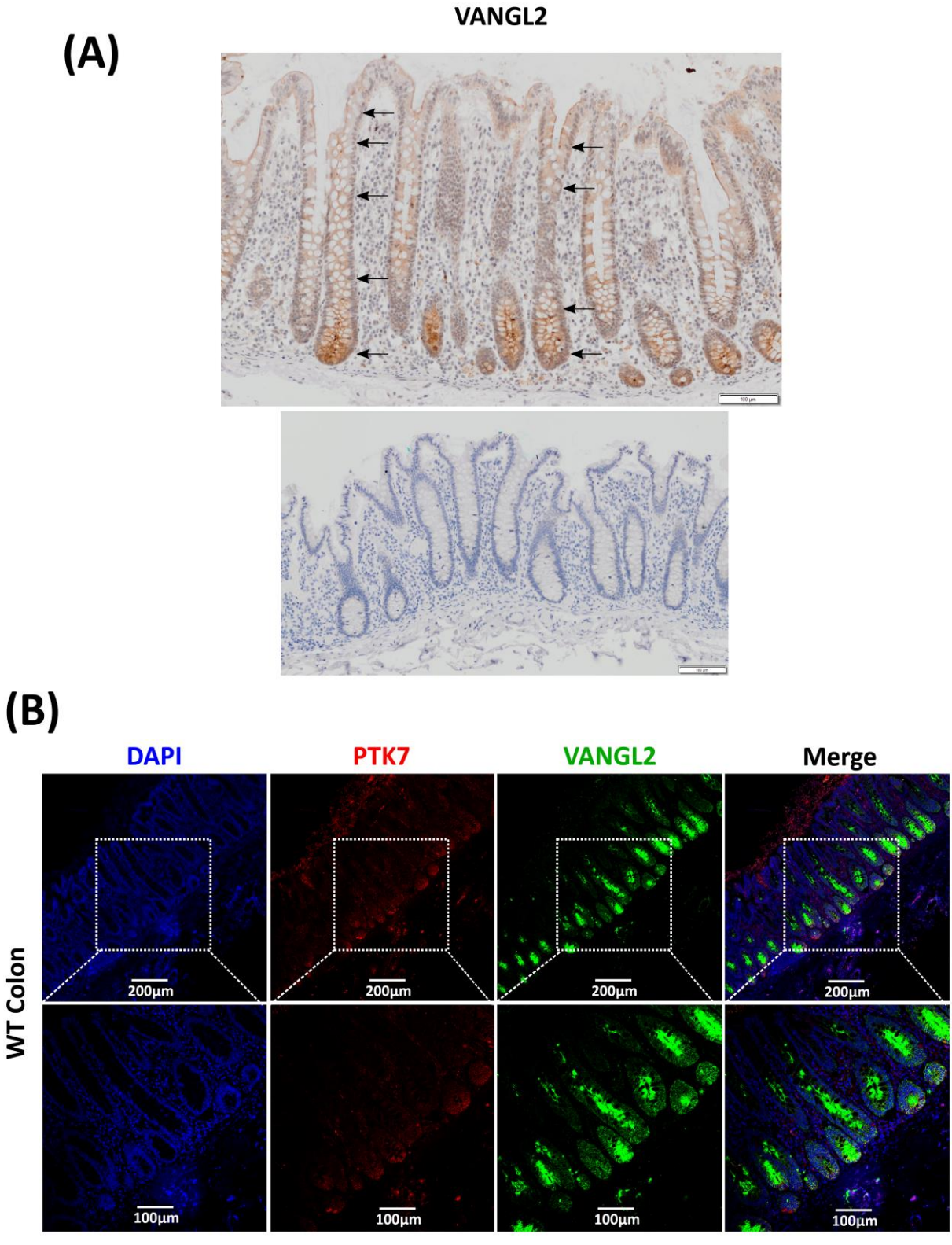


Figure 3.2.3 Localisation of VANGL2 within the human colon

(A) DAB staining of VANGL2 within a healthy human colon. Note the broad range of expression along the crypt axis, and largely cytoplasmic/membraneous localisation. Arrows indicate areas of expression. Bottom image is a negative control featuring no primary antibody. Image representative of 5 seperate stains **(B)** Immunofluorescent co-staining of VANGL2 and PTK7 in the healthy human colon. Note in the higher power images the co-localisation of both proteins (indicated in yellow in the merged image) at the cellular junctions within the base of the crypts. Representative images used from 3 stains.

3.2.4 *VANGL2* in human CRC: expression segregation and structural variation

Certain subtypes of colorectal cancer (CRC) such as those with MSI are associated with *VANGL2* promotor methylation, which can suppress transcription of *VANGL2* (Piazzi et al., 2013). However, there is no analysis looking directly at transcriptional variation of *VANGL2* within human CRC. Given that we have characterised the expression of *VANGL2* within the human and mouse colon, we then sought to examine dysregulation of *VANGL2* expression in disease, in this case CRC. Transcriptional variation within canonical Wnt signalling components has already been shown to have prognostic significance, with Wnt-1, β-catenin and E-cadherin all implicated in CRC (Stanczak et al., 2011). In breast cancer, *VANGL2* transcriptional upregulation correlates with poorer metastasis-free survival (MFS) (Puvirajesinghe et al., 2016). Using The Cancer Genome Atlas (TCGA) dataset, it was found that *VANGL2* mRNA is upregulated in 35 (9.2%) of 379 of colorectal adenocarcinoma patient tumours (Articles, 2012; J. Gao et al., 2013). By stratifying patients based on *VANGL2* mRNA expression I found that these patients with high *VANGL2* expression have similar overall survival (OS) to those with unaltered *VANGL2* expression (median of 43.1 months OS versus 48.2 months) (Figure 3.2.4). However, by stratifying disease free survival (DFS) based on mRNA upregulation, it was found that the patients with

upregulated *VANGL2* expression had significantly worsened median DFS (median of 34 months DFS) than those with unaltered expression (84 months).

Given the interesting findings from looking at this '*VANGL2*-high' subset of patients, I also searched for changes in expression of tumour-regulating genes within this subset. I found that the prevalence of MAPK1 (also known as ERK2) downregulation in the '*VANGL2*-high' patients is increased over '*VANGL2*-normal' patients. Therefore, VANGL2-high CRC patients have a significant association with downregulation of MAPK1 (OR = 2.616, 95% CI 1.155-6.12). MAPK1 is associated with promotion of cell growth and inhibition of apoptosis *in vitro* in CRC cells, while a similar effect has been shown in prostate cancer *in vivo* (Q. guang Chen et al., 2016; N. Zhang, Lu, & Chen, 2016). While this research showing MAPK1 promotes tumour malignancy does not fit with *VANGL2*-high patients showing MAPK1 downregulation, there is evidence to show that activation of MAPK1 in certain contexts can cause cell cycle arrest through induction of p21 and inhibition of Cyclin-dependent kinase 2 (Cdk2) (J. Han, Tsukada, Hara, Kitamura, & Tanaka, 2005; Malumbres et al., 2000). It could be speculated that this represents a mechanism through which downregulation of MAPK1 may promote CRC development, which may explain why we see decreased MAPK1 expression in patients with reduced DFS. However, as no direct link can be made, this association may be a result of a secondary effect from increased *VANGL2* expression.

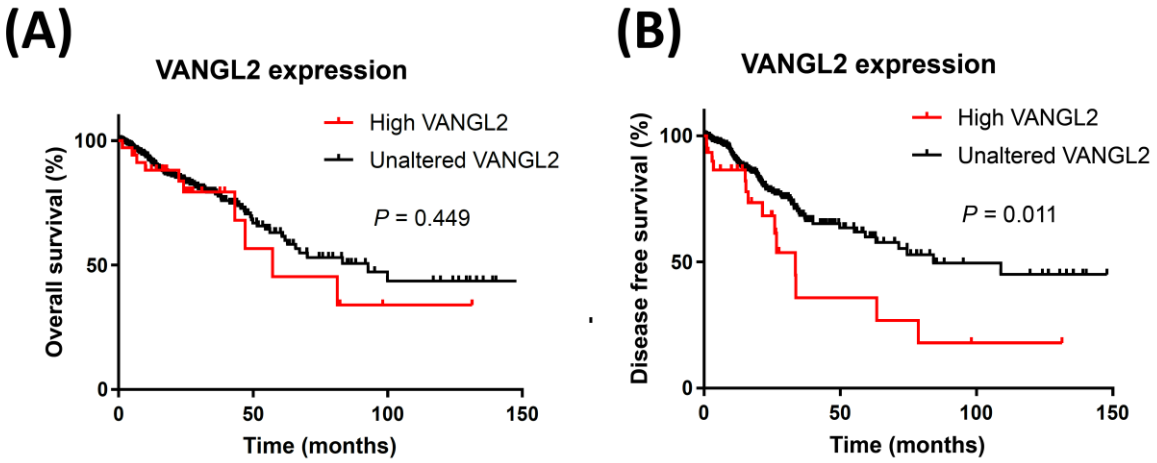


Figure 3.2.4 VANGL2 expression segregation in human CRC

Kaplan-Meier curve for **(A)** overall survival (OS) and **(B)** disease-free survival (DFS) of colorectal adenocarcinoma patients with high and unaltered expression of VANGL2. RNA expression data was obtained from TCGA database. Colorectal adenocarcinoma patients were stratafied based on high and unaltered VANGL2 tumour RNA expression. There is no significant difference in OS as a result of VANGL2 overexpression. High VANGL2 has a reduction in DFS (Log-rank test, P = 0.011). High VANGL2 median DFS = 33.5 months. Unaltered VANGL2 median DFS = 84.2 months. *N* = 379. The results here are based upon data generated by TCGA Research Network: http://cancergenome.nih.gov/ (data accessed 10.08.17)

	MAPK1 downregulation frequency (%)	MAPK1 no downregulation frequency (%)	OR	95% CI	P-value
VANGL2 tumour expression					
Upregulated (n = 35)	8 (22.86%)	27 (77.14%)	2.616	1.155-6.12	0.0436
Not upregulated (n = 344)	35 (10.17%)	309 (89.83%)			

OR - Odds Ratio; CI - 95% Confidence Interval; p - probability of Chi-square test

Table 3.2.4 High VANGL2 expression is associated with MAPK1 downregulation in CRC

Colorectal adenocarcinoma patients with upregulation of VANGL2 are significantly more likely to have MAPK1 downregulation. RNA expression data was obtained from the TCGA database. *N* = 379. The results here are based upon data generated by TCGA Research Network: http://cancergenome.nih.gov/ (data accessed 10.08.17)

3.2.5 *VANGL2* protein localisation and quantification in human CRC

Given that high expression of *VANGL2* is associated with worsened disease-free survival in colorectal cancer, further investigation into the role *VANGL2* plays in CRC is warranted. Using sections of human adenocarcinoma and non-cancerous tissue, I performed immunohistochemical staining for *VANGL2*. Intensity of expression of *VANGL2* is highly varied between patient tumour samples, illustrating that there is an opportunity to define patient subsets based on *VANGL2* expression levels (Figure 3.2.5A).

I then repeated this staining on tissue microarrays (TMAs) featuring cores taken from human colon cancers with matched healthy tissue cores, totalling 84 cases (Figure 3.2.5B). Overall core protein expression of *VANGL2* was quantified and cores stratified based on non-cancer or cancer tissue, patient age, sex, tumour grading and TNM staging data. No significant difference in *VANGL2* expression was found between non-cancer and cancerous tissue, nor was *VANGL2* expression different between CRC patients aged under 60 and those 60 and over. In assessing differences in gender, *VANGL2* expression was increased in females over males. We also see that *VANGL2* expression differs between tumour sub-types. Patients with tumours that are histologically grade II are found to have higher expression of

VANGL2 than those with grade I tumours, showing an association of higher *VANGL2* expression with tumour abnormality. While *VANGL2* expression was increased in Grade III tumours over grade I, this was not to a significant extent. No significant difference in expression was found between grade II and III tumours. While grading assesses the normality and differentiation of a growth, AJCC tumour staging defines a tumour's invasiveness into the surrounding tissue. Interestingly, T1 clinical stage cancers have significantly higher *VANGL2* expression than those patients with T2 stage cancers, indicating an association of higher *VANGL2* expression with tumours that are smaller in size or spread into the colon. While a decrease in *VANGL2* expression was also found when comparing T1 v T3 and T1 v T4 tumours, these were not to a significant extent. No association was found with *VANGL2* expression and lymph node spread. It is surprising that *VANGL2* is found higher in earlier stages given that it is associated with grade II tumours over grade I. However, these measures are assessing two different phenotypes of a tumour so it is possible that high *VANGL2* expression may confer higher tumour abnormality without increasing invasiveness.

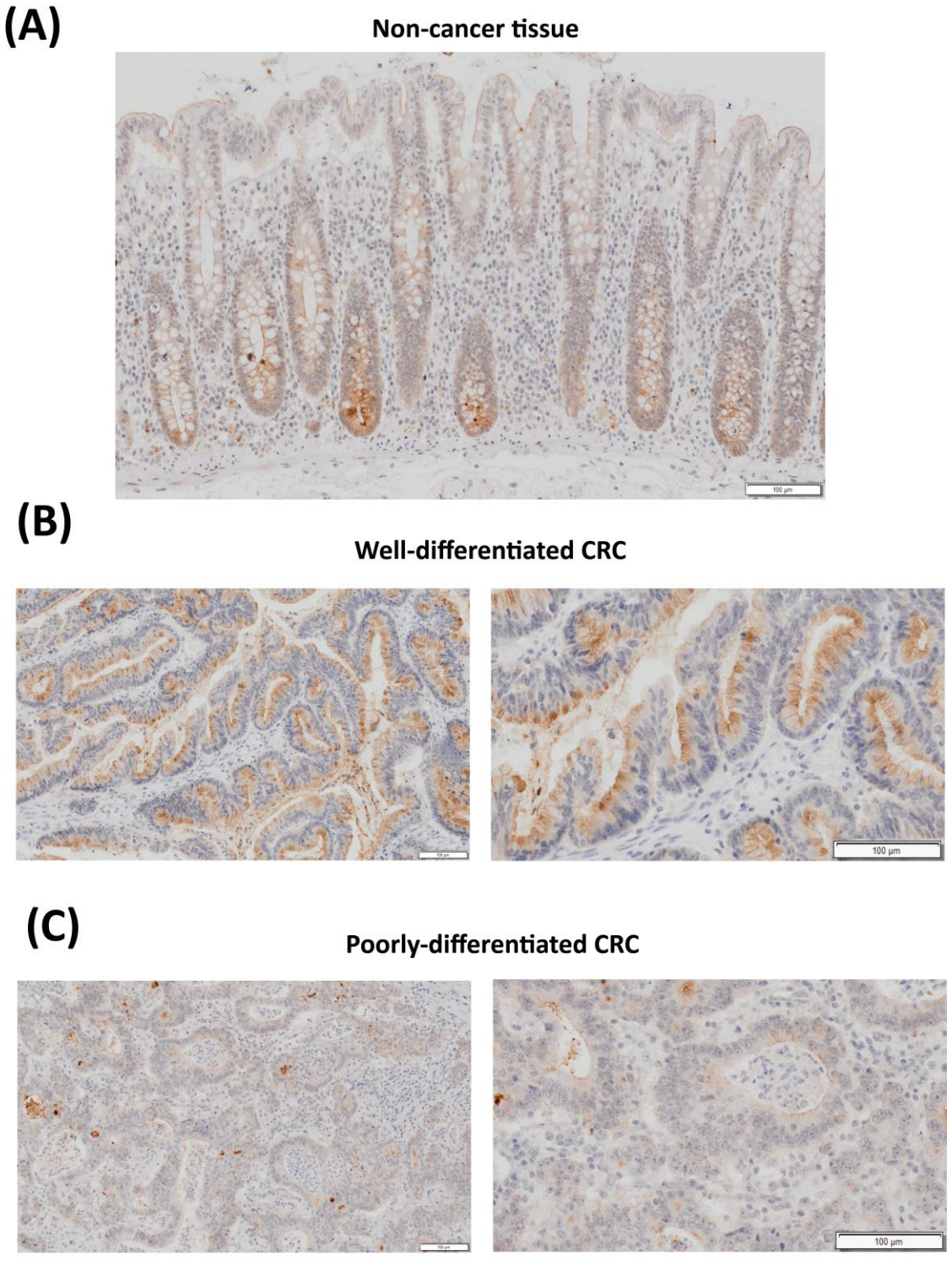


Figure 3.2.5A VANGL2 expression of varied intensity within CRC

Non-cancer colon tissue, well-differentiated CRC, and poorly-differentiated CRC tissue taken from patients was DAB stained for VANGL2 and imaged. Representative images used. **(A)** Shows comparitive non-cancer tissue. Image representative of 5 seperate stains. **(B)** Shows well-differentiated colorectal adenocarcinoma with high intensity VANGL2 staining. **(C)** Shows poorly-differentiated cancer with low intensity VANGL2 staining.

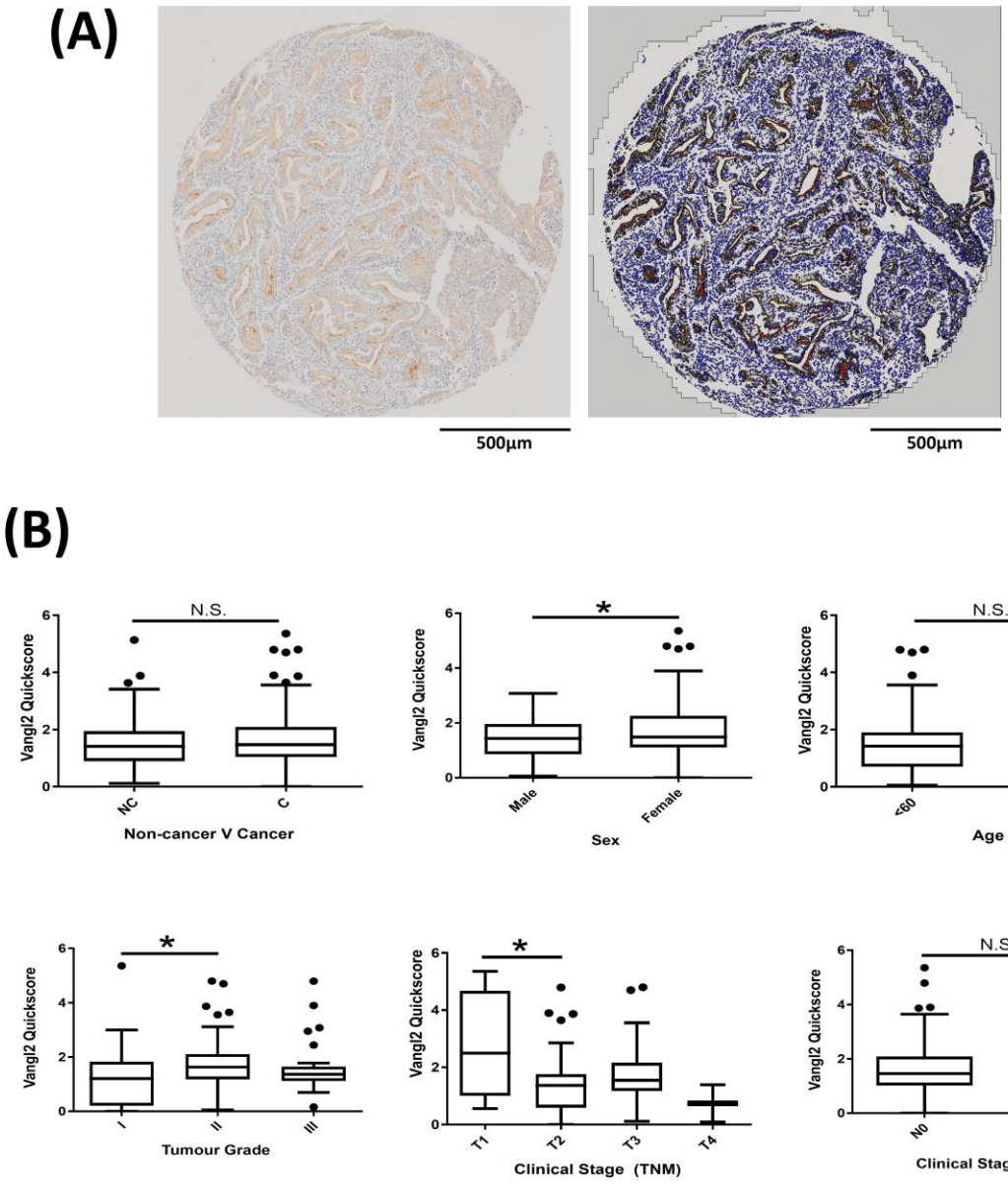


Figure 3.2.5B VANGL2 protein quantification in human CRC

Tumour tissue cores from colorectal cancer (CRC) patients on a tissue microarray (TMA) was DAB stained for VANGL2. **(A)** Representative image of VANGL2-stained cancer tissue core alongside annotated image of cellular analysis. Nuclei are identified in blue, while VANGL2 is categorised into low (yellow), moderate (orange), and high (red) intensity areas of expression. **(B)** VANGL2 quickscore expression shown in patient cores stratified by tissue type, sex, age, histological tumour grade, clinical tumour stage, and clinical lymph-node stage. Mean (SD); one way ANOVA. 84 patient cores were included in the analysis. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.3 Discussion

Many studies have supported a role for *VANGL2* in epithelial development. For example, *VANGL2* is required for directing migration and proper alignment of mouse corneal epithelial cells (Findlay et al., 2016). In other work, *VANGL2* is shown to be essential for cardiac morphogenesis (Ramsbottom et al., 2014) . Yates et al showed that *VANGL2* and fellow PCP gene Celsr1 are critical for foetal lung development (Yates, Schnatwinkel, et al., 2010) . However, there is also a growing field on the role of *VANGL2* and related PCP genes in homeostasis and disease (Poobalasingam et al., 2017; Vladar, Nayak, Milla, & Axelrod, 2016).

Transcriptional analysis found that *VANGL2* and several other PCP genes, such as WNT5A, are expressed within the adult mouse intestine, including the colon suggesting that non-canonical Wnt signalling could play a role here. It has previously been demonstrated that *VANGL2* is expressed during intestinal morphogenesis, while WNT5A has been shown to be essential for intestinal elongation during development (Cervantes et al., 2009; Elena Torban et al., 2007). My analysis shows that these genes and other 'core' PCP genes are also expressed in the healthy adult gut. Further to this, *VANGL2* protein is present within the adult mouse colonic epithelium, and is expressed in a broad manner across the crypt axis. Moreover, *VANGL2* is also

expressed within the cytosol and at cell-cell junctions. This shows that *VANGL2* is present in its typical functional location within the membrane of epithelial cells, as is seen during development (Ramsbottom et al., 2014; Seo, Habas, Chang, & Wang, 2017). We also see that WNT5A is expressed in the mouse colon, providing evidence of a sufficient ligand for PCP signalling, however in this study we have not directly demonstrated that Wnt5a is important for colonic non-canonical Wnt signalling in vivo. Additionally, PTK7 is seen expressed within the mouse colon, and seen co-localising with *VANGL2* within colonic crypts at cell-cell junctions. Interestingly, in the small intestine we do not see this co-localisation of *VANGL2* and PTK7, suggesting PCP components are not organised in the same way across the gut. The expression of several 'core' PCP components is shown transcriptionally and histologically indicating the presence of a 'signalling-hub' in the murine colon. This suggests that PCP signalling in the colon is possible and therefore may be important in maintaining epithelial homeostasis in the epithelium.

For the first time, it has been shown that *VANGL2* is also expressed in the human colon, in a similar broadly epithelial manner to the mouse. I also show that the human colon expresses PTK7 and the two proteins are seen to co-localise again at cellular junctions within the crypts, where the intestinal and colonic stem cells reside. This demonstrates that the murine colon is comparable to the human in PCP component expression and is a suitable model for analysing *VANGL2*'s role in

intestinal homeostasis and disease. As well as this, proof of *VANGL2*'s presence within the human colon raises the question of its importance in human disease. This is a question I address in this thesis. Further investigation of VANGL2 and PTK7 ability to bind in the colon would be of interest, as PTK7 has been shown previously to bind another PCP component, ROR2, in vertebrate Wnt signalling (Martinez et al., 2015).

Deregulation of several Wnt/PCP signalling components have already been shown to modulate CRC development. Overexpression of PTK7 and ROR2 both result in increased tumourigenesis (Lhoumeau et al., 2015; Mei et al., 2014). WNT5A has evidence also linking it to increased tumourigenesis, while other data indicates WNT5A acts as a tumour suppressor (Bakker et al., 2013; Cheng et al., 2014). Although *VANGL2* has been previously identified as a marker of poor prognosis in other cancers (such as breast cancer), the only previous analysis looking at *VANGL2* expression in CRC has been in cell lines, where it was found overexpression led to decreased proliferation, colony formation and β-catenin levels (Piazzi et al., 2013; Puvirajesinghe et al., 2016). This data suggests that *VANGL2* may act as a tumour suppressor in CRC. However, it must be acknowledged that there is only limited scope for extrapolation of cell line data to *in* vivo systems. As such, I present contrasting evidence in this chapter where elevated levels of *VANGL2* are associated with shortened time to recurrence in CRC following primary treatment, suggesting that *VANGL2* may not act as a tumour suppressor *in vivo* but may act like an oncogene

or possibly compound the oncogenic phenotype. No difference in overall survival was found between altered and unaltered patients. Therefore, high *VANGL2* tumour expression is associated with an increased risk of CRC recurrence without significantly lowering survival. I also find that high *VANGL2* tumour expression is associated with MAPK1 downregulation in in CRC patients. This finding would be consistent with research showing *VANGL2* downregulation via methylation associates with overactivation of the MAPK pathway (via oncogenic mutation in BRAF) (Piazzi et al., 2013). MAPK1 is commonly thought of as an oncogene, with research showing downregulation of MAPK1 can suppress cancer cell growth in colorectal and prostate cancer (Blaj et al., 2017; Q. guang Chen et al., 2016). However, there is also data indicating MAPK signalling can confer tumour-suppressive behaviour, including in colorectal cancer (Deschênes-Simard et al., 2013; Gulmann et al., 2009), as such, it remains unclear whether MAPK1 contributes to tumour growth or repression of such in vivo.

VANGL2 expression in human tumours could be linked with disease severity, and I found that in a cohort of CRCs there was a prominent level of *VANGL2* protein variability between tumours, which allows histological stratification to be performed. No association is seen between *VANGL2* protein expression and cancer and non-cancer tissue, showing that *VANGL2* levels cannot be used as a marker of cancer and suggesting that higher levels of *VANGL2* are not sufficient for predicting CRC

initiation. Evidence from *VANGL2* stratification shows that *VANGL2* expression is significantly higher in Grade II tumours over Grade I, suggesting that higher expression of *VANGL2* is associated this step up in architectural dysregulation in tumours, where the cells do not look like the epithelial cells from which the tumour arose. This association could be as a result of higher VANGL2 expression causing a propensity for tumours be grade II rather than grade I, or this effect could be a consequence from tumours that are grade II having a higher VANGL2 expression as a result. Interestingly, we see that higher *VANGL2* expression is associated with tumours of the smallest size and/or spread in human CRC.

Overall, these data provide an initial characterisation of the expression of *VANGL2* and non-canonical components in the human, and in the mouse to allow further investigation into the role of *VANGL2* in the intestine

Chapter 4: *VANGL2*-mediated signalling is implicated in the regulation of the ECM

4.1 Introduction

The data discussed in chapter 3 focused on the primary characterisation of *VANGL2* expression in the mouse and human intestine, as well as in human colorectal cancer. This revealed that *VANGL2* and other WNT/PCP components are expressed in the intestinal epithelium, and that a *VANGL2*-overexpressing subset of patients experience worsened disease-free survival (DFS).

The presence of *VANGL2* in the adult intestinal epithelium is in addition to its presence in the developing intestinal tube and other epithelial tissues (Cervantes et al., 2009; Iliescu et al., 2011). *VANGL2* has previously been identified as a requirement for normal lung architecture, while later it was implicated in maintenance adult lung homeostasis (Poobalasingam et al., 2017; Yates, Schnatwinkel, et al., 2010). It was shown that the heterozygous *Looptail* mutation (creating mislocalised *VANGL2* protein) created enlarged alveolar airspace decreased

lung function, possibly caused by altered distribution of actin microfilaments. *In vitro* assays identified reduced cellular migration in *VANGL2*-knockdown alveolar epithelial cells. Interestingly, the *VANGL2*-deficient lungs display markers of tissue damage in the form extracellular matrix remodelling components, with an altered macrophage population (enriched in large, highly vacuolar cells), significant increase in MMP12 expression, and abnormal elastin (which is a MMP12 substrate) deposition. *VANGL2* polymorphisms interact with lung function in response to insult (smoking), indicating that *VANGL2* plays a role in wound healing in the lung epithelium. Many of the studies that have defined the role of VANGL2 have been defined in development. *VANGL2*-knockdown in *Xenopus,* in both development and in tail regeneration in the adult, leads to aberrant neuronal length through loss of control of neural tissue proliferation. It is suggested that *VANGL2* and PCP signalling plays a role in signalling to terminate proliferation in neuroepithelium following tissue patterning. (Beane, Tseng, Morokuma, Lemire, & Levin, 2012). These data provide evidence that tissues where *VANGL2* is shown to be important developmentally often are also reliant on *VANGL2* for homeostatic mechanisms in the adult.

It is not known how *VANGL2* may be regulating homeostasis in the intestinal epithelium. Piazzi et al found *VANGL2* overexpression in CRC cells reduced proliferation concurrently with β-catenin activation (Piazzi et al., 2013). *VANGL2* has also been implicated in proliferative *VANGL2*-JNK signalling in breast cancer

(Puvirajesinghe et al., 2016). Given this evidence, and given *VANGL2* has been implicated in antagonising proliferative-driver canonical Wnt signalling, *VANGL2* may be mediating epithelial proliferation in the intestine (Shafer et al., 2011).

4.2 Results

4.2.1 *VANGL2*-deficient colonic epithelium is histologically normal

To assess *VANGL2*-signalling and its effect on the intestinal epithelium, I wanted to examine the adult intestine when *VANGL2* is lost. To do this I created an inducible, intestinal epithelial specific knockout of *VANGL2*. *Villin-Cre^{ERT}* transgenic mice have a tamoxifen inducible *Cre* recombinase downstream of the *Villin1* promoter, which is expressed specifically in the intestinal epithelium. *VANGL2$^{flox/flox}$* (provided by Professor Deborah Henderson at Newcastle University) transgenic mice have LoxP recombination target sites flanking exon 4 (which includes the transmembrane domains) of both copies of *VANGL2* (Figure 4.2.1D) (Ramsbottom et al., 2014). Cre activation in cells containing *VANGL2$^{flox/flox}$* leads to Cre-induced excision of exon 4 and leads to a premature stop codon, giving rise to a truncated *VANGL2* protein of 8 KDa, lacking its four transmembrane domains as well as its C-terminal PDZ binding domain required for its interaction with other proteins including Dvl1/2/3, Prickle1/2, and p62-SQSTM1 (Nagaoka, Tabuchi, & Kishi, 2015; Puvirajesinghe et al., 2016; Elena Torban et al., 2004). These mice were crossed to create *Villin-Cre^{ERT} VANGL2$^{flox/flox}$* mice. Upon exposure to tamoxifen, *Villin*-expressing cells will lose both functional copies of *VANGL2* in intestinal epithelial cells upon exposure to tamoxifen. Likewise, *Villin-Cre^{ERT} VANGL2$^{flox/WT}$* mice were created and lose one functional copy of *VANGL2*

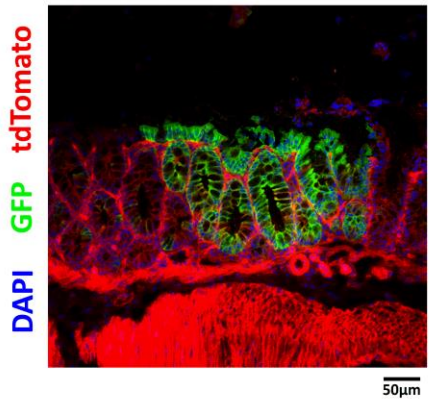
in epithelial cells upon induction. *Villin-Cre^{ERT} VANGL2$^{WT/WT}$* mice, hereafter known as *WT* for analysis purposes, lose neither copy of the gene upon induction and *VANGL2* remains that of the wild-type (WT). Given the well-known *VANGL2*Lp (S464N) mouse has a phenotype when a single copy of *VANGL2* is mutated, Vangl2 *WT* mice, with VillinCreERT activated, will act as a control for homozygous and heterozygous deletion of *VANGL2*.

Initially, the recombination efficiency of our *VANGL2* LOF model was assessed. The transgenic reporter, *Rosa$^{mT/mG}$* enables fluorescent detection of *Cre* activity, with red (tdTomato protein) fluorescence in cells which have seen no *Cre* expression, and green (GFP protein) fluorescence in cells which have seen *Cre* expression and therefore recombination activity. *Villin-Cre^{ERT} Rosa$^{mT/mG}$* adult mice were induced with intra-peritoneal injections of tamoxifen (80 mg per kg of bodyweight) on two consecutive days. On the 5th day following the first injection, animals were culled and the colons were prepared for cryo-sectioning. Sections were prepared and native fluorescence observed under a microscope (Figure 4.2.1A). The number of total and GFP^{+} crypts was counted in ten fields of view (10x magnification) for two different mouse colons. Around 20% of crypts within the colon experienced recombination events. Next, *Villin-Cre^{ERT} VANGL2$^{WT/WT}$ Rosa$^{mT/mG}$* and *Villin-Cre^{ERT} VANGL2$^{Flox/Flox}$ Rosa$^{mT/mG}$* adult mice were induced and culled as performed previously. Epithelial cells were isolated as described out in section 4.2.3, and cellular fluorescence was

analysed by flow cytometry (Figure 4.2.1B). $GFP^{+}tdTomato^{-}$, $GFP^{+}tdTomato^{+}$, and $GFP^{-}tdTomato^{-}$ populations were all identified (Figure 4.2.1B). Both *VANGL2*$^{WT/WT}$ and *VANGL2*$^{Flox/Flox}$ genotypes had GFP^{+} populations of similar size, with 42.55 and 34.6%, respectively (Figure 4.2.1C) (P = 0.4281) (n = 5). As the proportion of recombined cells in the *VANGL2* LOF epithelium is similar to the WT, I can assert that loss of *VANGL2* does not alter epithelial survival in the adult colon. As five days following induction is quite a short time-frame, analysis of longer-term induction of loss of VANGL2 may produce a higher efficiency.

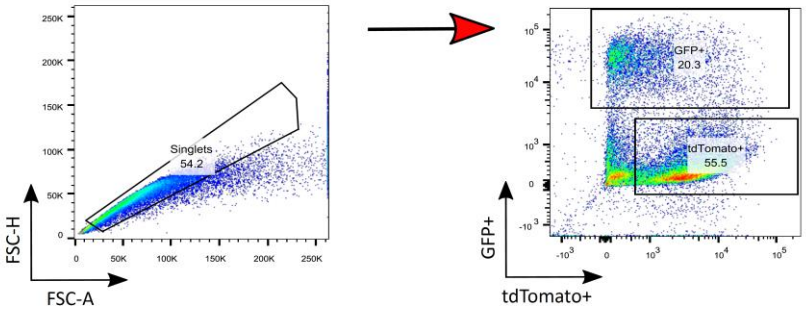
Villin-Cre^{ERT} *VANGL2*$^{WT/WT}$ and *Villin-Cre*^{ERT} *VANGL2*$^{flox/flox}$ adult mice were given intra-peritoneal injections of tamoxifen (80 mg per kg of bodyweight) on two consecutive days. On the 5th day following the first injection, animals were culled and intestines harvested for subsequent analysis. During the experiment and at the timepoint mice were culled, mice exhibited no altered behaviour or signs of injury between genotypes, and intestines and other tissues were macroscopically normal. *Villin-Cre*^{ERT} *VANGL2*$^{WT/WT}$ and *Villin-Cre*^{ERT} *VANGL2*$^{flox/flox}$ colons were fixed and histologically evaluated with a haematoxylin & eosin (H&E) stain (Figure 4.2.1E). No differences between genotypes were observed in colonic morphology and in inflammatory cell infiltrates.

(A)



	VillinCreERT mTmG	
	Colon 1	Colon 2
% GFP⁺crypts	23.4	16.3
% GFP⁺crypts (average)	19.9	

(B)



(C)

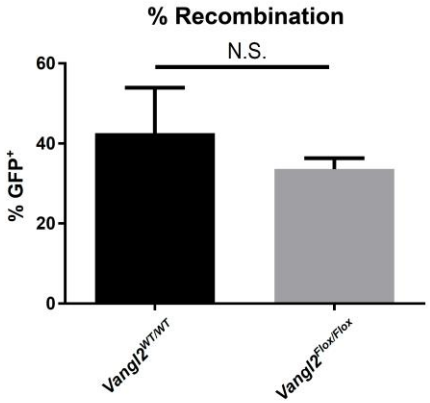
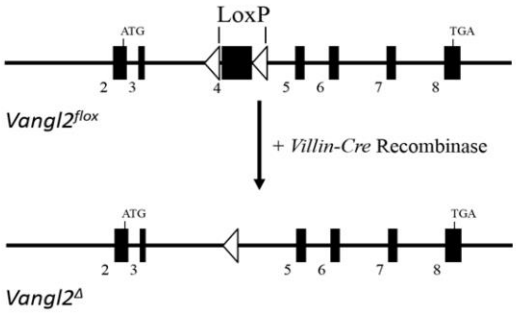


Figure 4.2.1 VANGL2-deficient colonic epithelium is histologically normal

(A) *Villin-Cre*^{ERT} *Rosa*$^{mT/mG}$mice were induced with tamoxifen. Cryosectioning was performed on the colons and native fluorescence observed to analyse *Cre* activity. Non-recombined epithelial cells express tdTomato (Red), recombined cells express GFP (Green), and sections were also stained for DNA with DAPI (Blue). **(B)** *Villin-Cre*^{ERT} *Rosa*$^{mT/mG}$ flow cytometry gating strategy. *Villin-Cre*^{ERT} *Rosa*$^{mT/mG}$ epithelial cells were isolated from a mouse colon as described in section 4.2.2. Single cells were analysed by flow cytometry and gated for singlets; and GFP/tdTomato positivity. tdTomato^{+} cells are non-recombined; whereas GFP^{+} cells have experienced *Cre*-mediated recombination. Representative analysis from two seperate experiments. **(C)** Percentage recombination in *Villin-Cre*^{ERT} *Vangl2*$^{WT/WT}$ *Rosa*$^{mT/mG}$ and *Villin-Cre*^{ERT} *Vangl2*$^{Flox/Flox}$ *Rosa*$^{mT/mG}$ epithelial cells. The proportion of GFP^{+} cells in the total GFP^{+} or tdTomato^{+} population was calculated from flow cytometer counts. n = 5. Mean (SD); students t-test. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

(D)



(E)

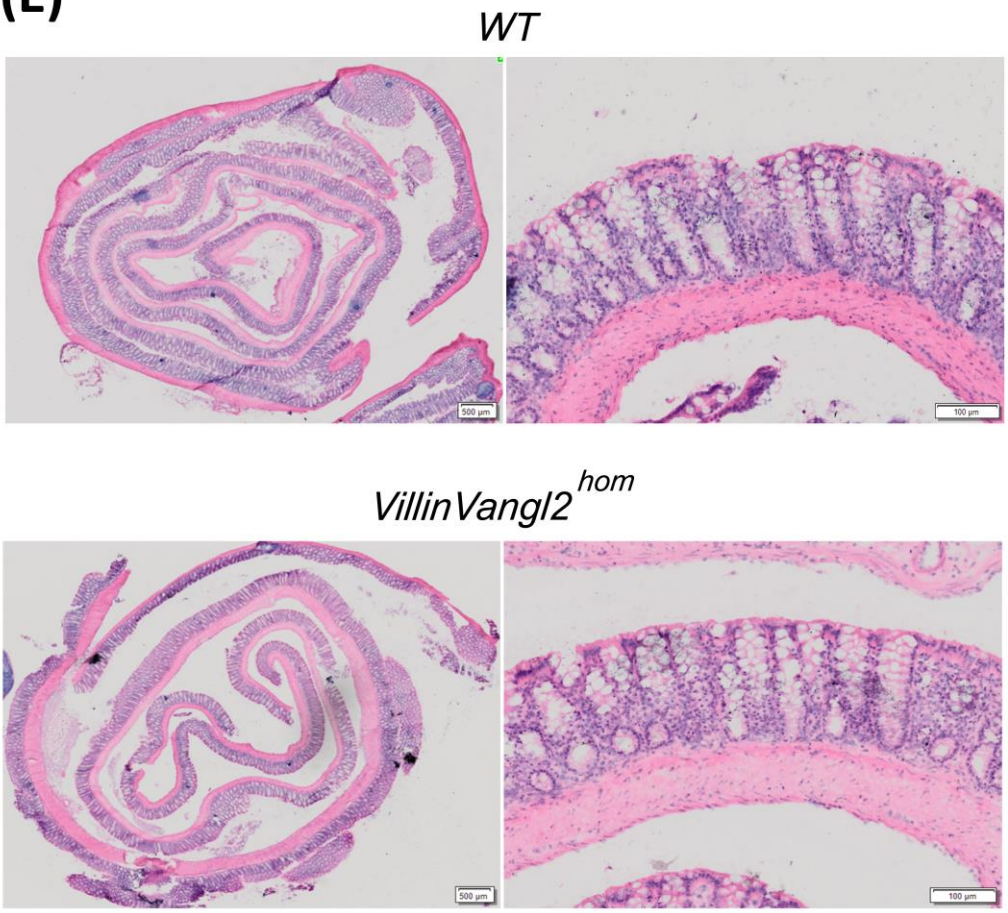


Figure 4.2.1 VANGL2-deficient colonic epithelium is histologically normal - *continued*

(D) Diagram outlining Cre-induced recombination of VANGL2 exon 4 in the mouse intestinal epithelium. *Villin-Cre^{ERT} VANGL2$^{WT/WT}$* or *Villin-Cre^{ERT} VANGL2$^{flox/flox}$* mice were induced with intraperitoneal injections of tamoxifen 5 days prior to culling. **(E)** H&E of *WT* and *Villin Vangl2^{hom}* floxed mouse colons (representative images for 5 mice from each genotype). Colons harvested and fixed five days following induction with tamoxifen, and then H&E stained. Low- and High- power microscopy images shown.

4.2.2 Loss of *VANGL2* in the colonic epithelium has no effect on proliferation

I then assessed the amount of proliferation within the colon of the *Villin-Cre^{ERT} VANGL2$^{WT/WT}$* and *Villin-Cre^{ERT} VANGL2$^{flox/flox}$* mice. As *VANGL2* has previously been implicated in regulating proliferation in colonic epithelial cells. While no obvious epithelial thickening was present in either genotype (which would indicate epithelial hyperplasia or hypoplasia), a quantitative method is necessary to identify low-level proliferative changes or increased proliferation to maintain homeostasis (i.e. from decreased epithelial viability).

Fixed colonic tissue from the genotypes *VANGL2$^{WT/WT}$*, *VANGL2$^{Flox/WT}$*, and *VANGL2$^{Flox/Flox}$* was stained for Ki67 or Proliferating cell nuclear antigen (PCNA) (Figure 4.2.2) to evaluate proliferation in these mutant tissues. Ki67 is a marker only expressed in cycling cells (in G1, S, G2 and M phases). PCNA is a protein required for DNA synthesis expressed in cells in late G1 and S phases. While both stains routinely used for identifying proliferative nuclei, there is some evidence that suggests Ki67 is the more specific marker of cellular proliferation (Bologna-Molina, Mosqueda-Taylor, Molina-Frechero, Mori-Estevez, & Sánchez-Acuña, 2013). Using computational analysis, the number of positively- and negatively-stained nuclei within the colonic

epithelium was quantified and the percentage of positive nuclei was calculated for each mouse colon. I, using Definiens Tissue Studio, trained the software to identify haematoxylin-stained nuclei, and DAB-stained nuclei within the epithelium of microscopy images. The trained parameters for nuclei identification was then applied to all imaged slides for analysis. I find that there is no significant change in percentage of positive Ki67 or PCNA epithelial nuclei across genotypes.

Therefore, *VANGL2* loss in the colonic epithelium has no effect on proliferation. Previously published work shows an observed decrease in proliferation when *VANGL2* is knocked down in breast cancer cells (Puvirajesinghe et al., 2016). In contrast, in CRC cells *VANGL2* overexpression is shown to decrease proliferation (Piazzi et al., 2013). In the developing lung, *VANGL2*$^{Lp/Lp}$ lungs have no alteration in proliferation, while the same genotype in the developing kidney displays decreased proliferation at E18.5 but not at E14.5 (Yates, Papakrivopoulou, et al., 2010; Yates, Schnatwinkel, et al., 2010).

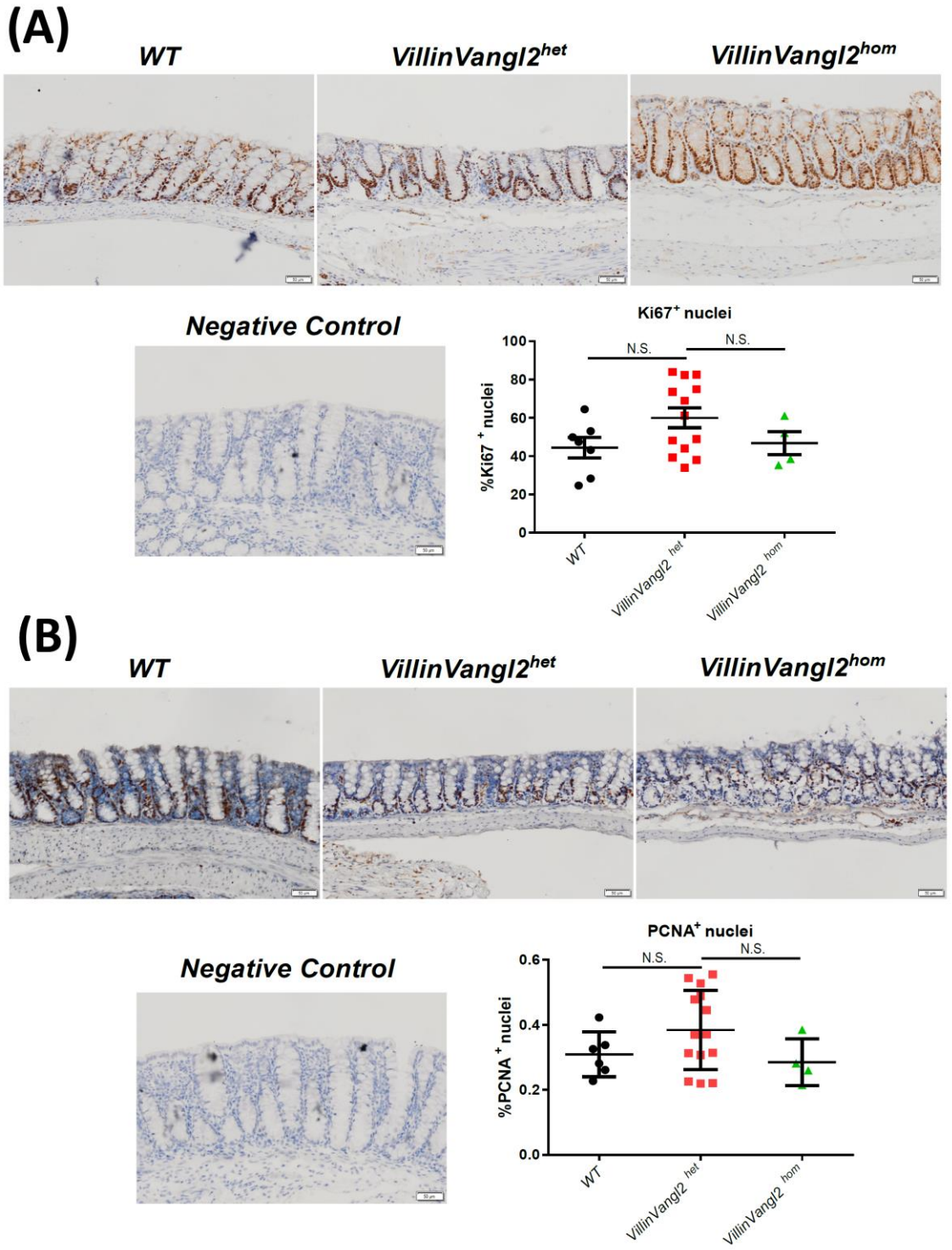


Figure 4.2.2 Loss of VANGL2 in the colonic epithelium has a neutral effect on proliferation

Loss of VANGL2 has a neutral effect on the number of **(A)** Ki67 and **(B)** PCNA positive nuclei in the colon. *Villin-Cre Vangl2^{flox}* mice were induced for 5 days with intraperitoneal injections of tamoxifen. Harvested colons were fixed and stained for Ki67/PCNA and DAB visualised and counterstained with haematoxolin. Negative controls are stained minus primary antibody. Resultant stains were scanned and number of DAB positive and negative nuclei was quantified using Definiens Tissue Studio. Percentage positive nuclei was calculated for each mouse and is presented as mean (SD). One-way ANOVA. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. $N = 4$.

4.2.3 Isolation of the colonic epithelium

Given that I was unable to find a proliferative phenotype with regards to *VANGL2*-mediated signalling, I decided to evaluate transcriptional changes in epithelial cells that are deficient in *VANGL2*. Epithelial cells are only a small proportion of the total cells in the colon, therefore it was necessary to enrich tissue samples for epithelial cells to avoid missing subtle transcriptional changes. Previously published protocols involved a lengthy and/or complicated method, or were found to be ineffective on the colonic epithelium (Nik & Carlsson, 2013; Wong et al., 2015). I therefore decided to develop a protocol using Ethylenediaminetetraacetic acid (EDTA) to extract epithelial cells from a whole colon. This is a similar technique to what is used to extract intestinal crypts for purposes of intestinal organoid culture (Sato et al., 2009). First, colons are harvested, flushed with PBS and cut into small pieces (1-2 mm). This colonic tissue is incubated with 25 mM EDTA for 30 minutes and then the tissue is agitated with a pipette in fresh PBS, with the cells collected in the PBS constituting one 'fraction'. Four fractions are taken in total and contents combined to give homogenised epithelium extracted from the colon, which can be subsequently used for transcriptional analyses (Figure 4.2.3A). I validated this protocol for producing enriched epithelial fractions by looking at intestinal epithelial and mesenchymal marker transcripts. We can see that the crypt epithelial stem cell marker Lgr5 is highly enriched in my epithelial fractions when compared to expression from the

whole colon. We also see moderate Krt19 (pan-epithelial marker) expression across epithelial fractions. Importantly, we see almost no mesenchymal marker expression (αSMA) in the epithelial fractions. These data validate this method for producing pure epithelial cell extracts for the purposes of transcriptional analysis.

Having developed a reliable method for extracting epithelial cells, I used this to isolate epithelial cells from the *VANGL2*-knockout mouse colon described previously, in order to validate loss of functional *VANGL2* transcription (Figure 4.2.3B). The *Villin-Cre^{ERT} VANGL2$^{WT/WT}$* and *Villin-Cre^{ERT} VANGL2$^{flox/flox}$* mice were induced as previously shown and culled after 5 days. Epithelium was extracted using the above method and mRNA was extracted from the pooled fractions. cDNA synthesised from the transcripts were used in a qRT-PCR analysis assaying *VANGL2* expression in the colonic epithelium. I found that *VANGL2* mRNA expression was significantly reduced in the *Villin-Cre^{ERT} VANGL2$^{flox/flox}$* epithelium versus the *WT* controls. While this validates that *VANGL2* expression in the colonic epithelium is lost, the data also indicates that Cre mediated recombination of *VANGL2* is not 100% efficient, which has previously been reported (Balzola, Bernstein, Ho, & Lees, 2010). Subsequently, data generated from transcriptional analysis should be analysed with the caveat that there will also be contaminating epithelial cells which have not lost *VANGL2*.

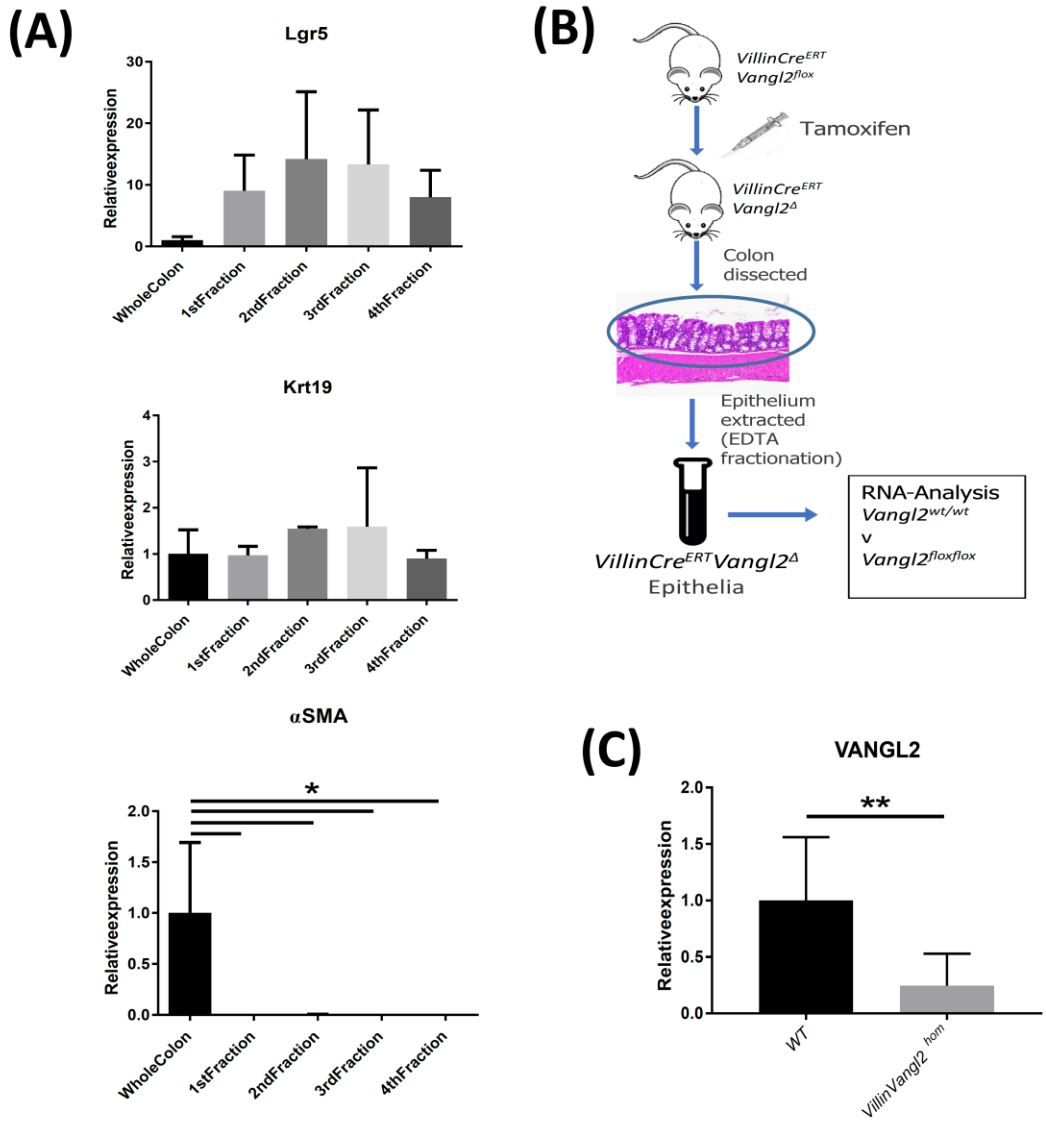


Figure 4.2.3 Isolation of the colonic epithelium

(A) Mouse colons were harvested and epithelial cells extracted using EDTA fractionation. RNA extraction and subsequent cDNA synthesis was performed from epithelial fractions and from the whole colon for protocol validation. qPCR was performed for epithelial markers Lgr5 and Krt19, and for mesenchymal marker αSMA. Expression presented relative to expression of each marker in the whole colon. **(B)** Diagram illustrating methodology for extracting colonic epithelium from the induced *Villin Vangl2*^{WT} and *Villin Vangl2*^{hom} mice for use in subsequent RNA analyses **(C)** qRT-PCR analysing colon epithelial RNA expression of VANGL2 in the *Villin Vangl2*^{WT} and *Villin Vangl2*^{hom} mouse. Epithelial tissue was extracted from harvested mouse colons by EDTA fractionation. RNA was extracted from resultant epithelium and cDNA synthesised. qRT-PCR was then performed targeting VANGL2. Expression presented relative to expression of VANGL2 in the WT. All expression normalised to Ppia. Mean (SD) values shown. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

4.2.4 Transcriptome analysis of *VANGL2*-deficient epithelial cells

Since previous work has indicated *VANGL2* may participate in Wnt/PCP-mediated inhibition of canonical Wnt signalling, I analysed canonical Wnt target gene expression in WT and in *VANGL2*-deficient epithelium (Piazzi et al., 2013; Shafer et al., 2011). Transcript expression of five canonical Wnt pathway target genes was analysed in *WT* and *Villin VANGL2*^{hom} epithelial tissue (Figure 4.2.4): Cyclin D1 (CCDN1), MYC Proto-Oncogene (cMYC), Lymphoid Enhancer Binding Factor 1 (LEF1), Axin 2 (AXIN2), and SRY-Box 9 (SOX9). No significant changes in canonical target gene expression was found in the *VANGL2*-deficient epithelial cells. This suggests that *VANGL2* does not play a role in regulating canonical Wnt signalling in the healthy colonic epithelium.

To identify pathways regulated by *VANGL2*, a transcriptomic approach was utilised. *Villin-Cre*^{ERT} *VANGL2*$^{WT/WT}$ and *Villin-Cre*^{ERT} *VANGL2*$^{flox/flox}$ epithelial tissue was collected as described previously. This tissue was then used for RNA-Sequencing. The datasets from each genotype were compared and genes that were upregulated or downregulated in the *Villin-Cre*^{ERT} *VANGL2*$^{flox/flox}$ were identified (Table 4.2.4A) compared to *VANGL2*WT. 38 genes were found to be significantly upregulated in the

Villin-Cre^{ERT} *VANGL2*$^{flox/flox}$ mouse, while 23 were found to be significantly downregulated ($q < 0.05$).

In the upregulated gene list, transcript GM22973 is expressed exclusively in the *VANGL2*^{hom} epithelium (Table 4.2.4A). This is a predicted small nuclear RNA (snRNA) of unknown function. TRPV6, is a multi-pass membrane protein that functions as a calcium channel, and is important for Ca^{2+} ion homeostasis in epithelial tissues. It is also upregulated in some cancers (Al-Ansary, Bogeski, Disteldorf, Becherer, & Niemeyer, 2010). Other Ca^{2+} signalling-related transcripts upregulated are ATP2B1, and CLCA4 (Agnel, Vermat, & Culouscou, 1999; Hilfiker, Strehler-Page, Stauffer, Carafoli, & Strehler, 1993; J.-B. Peng et al., 2000). These could be potential targets or regulators of the non-canonical Wnt/Ca^{2+} signalling pathway. It is possible that Wnt/Ca^{2+} activity could be upregulated in order to overcome the loss of *VANGL2* in the Wnt/PCP pathway.

Matrix Metallopeptidase 7 (MMP7) is also upregulated. MMPs are involved in the breakdown of extracellular matrix (ECM), and MMP7 is involved in wound healing in intestinal epithelial cells (Puthenedam et al., 2011). There are also several upregulated genes that are involved in the Rho/ROCK signal transduction pathway (which is a known downstream target of Wnt/PCP signalling). Rho GTPase Activating

Protein 5 (ARHGAP5), Rho Associated Coiled-Coil Containing Protein Kinase 1 and 2 (ROCK1 and ROCK2) are all upregulated when *VANGL2* is knocked out (Burbelo et al., 1995; Habas, Dawid, & He, 2003; Ishizaki et al., 1996; Takahashi, Tuiki, Saya, & Kaibuchi, 1999).

Of the genes significantly downregulated in the *VANGL2*-deficient epithelium, we interestingly see a calcium activated proteinase in CAPN13 (Dear & Boehm, 2001). There is also a large representation of ECM proteins downregulated when *VANGL2* is knocked out. The type 1 collagen COL1A2, and the type 6 collagen COL6A1 were identified, both of which are important for tissue integrity. Matrix-associated proteins Decorin (DCN), and Secreted Protein Acidic and Cysteine Rich (SPARC) have been implicated in regulation of tumour development. Chymase 1 (CMA1) was also found, and is produced by mast cells and is thought to be involved in the degradation of the ECM (Feng & Tang, 2014; Groulx et al., 2011; A. He & Shi, 2012; Järvinen & Prince, 2015; Sampurno et al., 2015). Two genes which are known to protect the intestinal epithelium from insult, Trefoil Factor 2 (TFF2), and Lysozyme 2 (LYZ2), were also identified as downregulated (Aamann, Vestergaard, & Grønbæk, 2014; Immunologists., Almaghrabi, Leger, & Caspi, 2017). Interestingly, Growth Arrest Specific 1 (GAS1) is known to play a role in growth suppression by blocking entry to S-phase, and is a putative tumour suppressor gene is also lost in *VANGL2*flox/flox colonic epithelium versus control (Evdokiou & Cowled, 1998).

To find out which cellular signalling pathways were upregulated or downregulated as a result of loss of *VANGL2*, I performed pathway analysis on the significantly changed gene lists identified in Table 4.2.4A. An over-representation analysis (using tools from ConsensusPathDB) was performed on each of the lists, which maps genes on the list to a databases of pathway-based gene sets (Kamburov et al., 2011; Kamburov, Wierling, Lehrach, & Herwig, 2009). A minimum of 2 matched genes was used for identification of a pathway and only pathways with a significant association were used ($q < 0.05$). The top 10 significantly over-represented pathways from the upregulated and downregulated gene lists are shown (Table 4.2.4B).

Pathways identified from genes upregulated in the *VANGL2* knockout (KO) include the Rho GTPase/ROCK pathway (ROCK1; ROCK2). The ROCK proteins, which control actin polymerisation, are activated by Rho GTPases which are utilised in Wnt/PCP signalling. As the deletion of *VANGL2* associates with the upregulation of ROCK transcript expression, it may be hypothesised that *VANGL2* acts to restrict the Rho GTPase/ROCK pathway in the colonic epithelium. Another pathway upregulated which uses the ROCK proteins is the focal adhesion pathway (ARHGAP5; ROCK1; ROCK2) which, again, functions through actin polymerisation regulation to control cell-ECM adhesion and interaction.

Pathways identified from downregulated genes include extracellular matrix organisation (DCN; CAPN13; CMA1; SPARC; COL1A2; COL6A1). The extracellular matrix is a network of biomolecules which provide numerous functions including protection against physical stress from compression, providing stiffness to tissue, as well as having effects on cellular migration, differentiation, and cellular programmes. The focal adhesion pathway also has components which are found to be downregulated (COL6A1; MYL7; COL1A2). These are ECM-based components which are involved in this process. Based on the changes seen in these pathways, it may be that *VANGL2* restricts Rho GTPase/ROCK pathway signalling. As this signalling controls cell-ECM interactions, these changes may explain why we see the reduction of expression of several ECM components, including type I Collagen (the most abundant in the ECM). We also see that transcriptional targets of FRA1 and FRA2 are also downregulated when *VANGL2* is lost (DCN; COL1A2). FRA1 and FRA2, both members of the Fos gene family, are AP-1 transcription factor subunits. FRA1 has been shown to be a regulator of epithelial-to-mesenchymal transition (EMT) in CRC cells, and is found at the invasive front in human colorectal cancers (Diesch et al., 2014). This suggests that FRA1/FRA2-dependent AP-1 activity is abrogated when *VANGL2* is lost in the colonic epithelium.

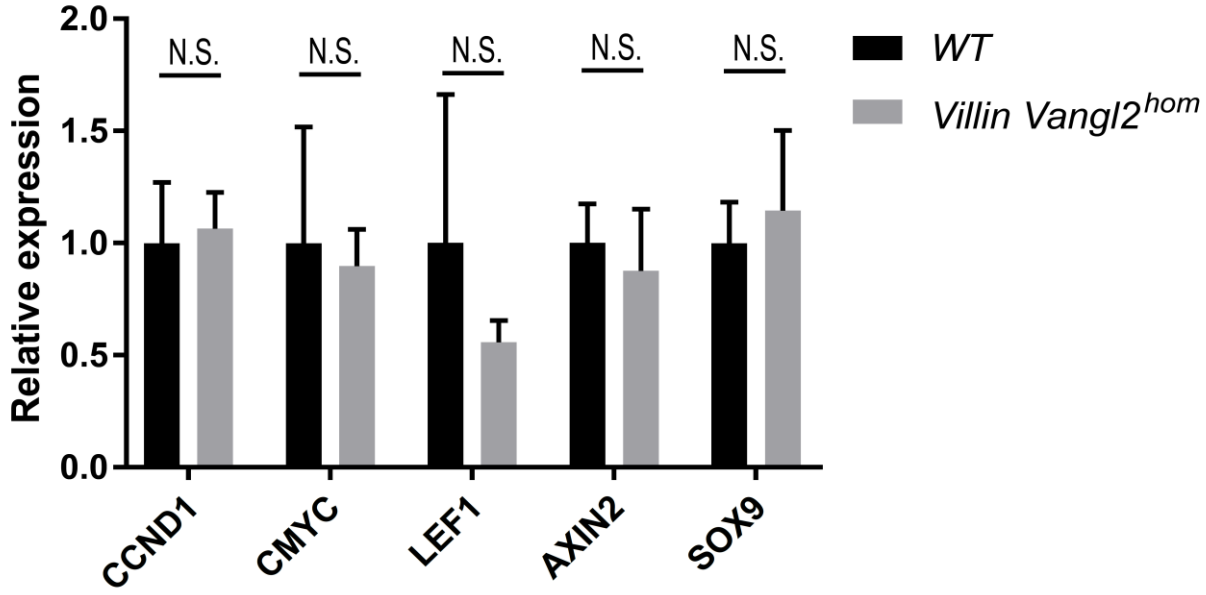


Figure 4.2.4 Canonical Wnt signalling target expression is unchanged following loss of VANGL2 in the colonic epithelium

qRT-PCR analysing colon epithelial RNA expression of VANGL2 in the *Villin Vangl2*^{WT} and *Villin Vangl2*^{hom} mouse. Epithelial tissue was extracted from harvested mouse colons by EDTA fractionation method shown previously. RNA was extracted from resulting epithelium and cDNA synthesised. qRT-PCR was then performed targeting CCND1, CMYC, LEF1, AXIN2, and SOX9. For each gene, expression values are relative to the *WT*. All expression normalised to Ppia. Mean (SD) values shown. Students t-test. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 4.2.4A RNA-Sequencing analysis of VANGL2-deficient colonic epithelium

The EDTA-fractionation method for collecting colonic epithelium RNA (outlined in Figure 4.2.3) was performed using *Villin Vangl2*^{WT} and *VillinVangl2*^{hom} mice. RNA-Seq expression analysis was carried out and comparison of the two datasets significantly altered genes is shown (q < 0.05). 38 upregulated genes and 23 downregulated genes were identified. Log2 fold changes were calculated using the relative FPKM for each gene. * = Transcript is found exclusively in the *Vangl2*^{hom} mouse

Upregulated in *Villin Vangl2*^{hom}	Fold Change (Log2)	q-value
Gm22973	*	0.032
Krtap13	2.382	0.020
Clca4b	2.372	0.020
Trpv6	2.178	0.020
Gm9765	1.651	0.020
Itln1	1.613	0.020
5033404E19Rik	1.370	0.020
Gm13225	1.132	0.020
Ifi202b	1.131	0.020
Slc30a10	1.112	0.020
Sprr1a	1.048	0.020
Reg3b	1.010	0.020
Adgrf1	0.949	0.020
Ctsf	0.827	0.020
Zfp820	0.817	0.043
Zfp947	0.778	0.032
Mmp7	0.722	0.020
Lrrcc1	0.708	0.032
Mgat4c	0.684	0.020
Emp1	0.682	0.020
Hmgn5	0.659	0.020
Zfp518a	0.659	0.020
Gm37697	0.658	0.032
Rock2	0.656	0.020
Zfp946	0.639	0.043
Lrrc19	0.635	0.020
Pof1b	0.635	0.020
Nxpe4	0.622	0.020
Clca4a	0.612	0.020
Gda	0.609	0.020
Slfn4	0.593	0.020
Arhgap5	0.590	0.020
Cebpd	0.572	0.043
Rock1	0.567	0.032
Samd9l	0.563	0.032
Ifit1	0.555	0.043
Smc6	0.551	0.032
Atp2b1	0.528	0.020

Downregulated in *Villin Vangl2*^{hom}	Fold Change (Log2)	q-value
Xlr4a	-3.693	0.032
Zfp865	-2.206	0.020
Stra6	-1.830	0.032
Cma1	-1.576	0.020
Tff2	-1.345	0.043
Col6a1	-1.272	0.020
Slpi	-1.164	0.020
Gm11942	-1.075	0.032
Gas1	-1.034	0.020
Naaladl1	-1.031	0.020
Col1a2	-0.987	0.020
Gm10073	-0.932	0.020
Arg1	-0.870	0.020
Lyz2	-0.855	0.020
Dcn	-0.840	0.020
Sparc	-0.838	0.043
Hbb-bs	-0.835	0.032
Capn13	-0.765	0.020
Myl7	-0.759	0.020
Ifi27l2a	-0.756	0.020
Hck	-0.723	0.020
H2-Aa	-0.721	0.020
Gstp2	-0.673	0.020

Table 4.2.4B Transcriptome pathway analysis of VANGL2-deficient colonic epithelium

Over-representation analysis of significantly upregulated and downregulated genes identified in Table 4.2.5A. Genes are mapped to pathway-based gene sets (with a minimum of 2 matched genes), and only pathways with a significant association (q < 0.05) were included. The top 10 significantly over-represented pathways are shown from the upregulated and downregulated genes. Analysis carried out using ConsensusPathDB (Kamburov, Wierling, Lehrach, & Herwig, 2009; http://cpdb.molgen.mpg.de/CPDB).

Pathway	Gene members upregulated
RHO GTPases Activate ROCKs	ROCK1; ROCK2
pkc-catalyzed phosphorylation of inhibitory phosphoprotein of myosin phosphatase	ARHGAP5; ROCK1
Sema4D induced cell migration and growth-cone collapse	ROCK1; ROCK2
rho cell motility signaling pathway	ARHGAP5; ROCK1
JAK-STAT	ROCK1; ROCK2
Focal Adhesion	ARHGAP5; ROCK1; ROCK2
RhoA signaling pathway	ROCK1; ROCK2
Focal adhesion - Homo sapiens (human)	ARHGAP5; ROCK1; ROCK2
C-MYB transcription factor network	ATP2B1; CEBPD
Integrin-mediated Cell Adhesion	ROCK1; ROCK2

Pathway	Gene members downregulated
Extracellular matrix organization	DCN; CAPN13; CMA1; SPARC; COL1A2; COL6A1
Integrin	DCN; COL6A1; COL1A2
Validated transcriptional targets of AP1 family members Fra1 and Fra2	DCN; COL1A2
Binding and Uptake of Ligands by Scavenger Receptors	SPARC; COL1A2
miRNA targets in ECM and membrane receptors	COL6A1; COL1A2
Focal adhesion	COL6A1; MYL7; COL1A2
ECM proteoglycans	DCN; SPARC
IL4-mediated signaling events	ARG1; COL1A2
Collagen biosynthesis and modifying enzymes	COL6A1; COL1A2
Collagen formation	COL6A1; COL1A2

4.2.5 Transcriptional investigation of *VANGL2*-mediated regulation of the ECM

Given the numerous altered genes identified with the pathway in figure 4.2.4, I decided to investigate the role *VANGL2* plays in regulating the extracellular matrix (ECM). The ECM maintains tissue integrity, regulates cellular differentiation, proliferation, and cell migration. It also contains a host of bound and soluble growth factors, such as VEGF, which can be found bound to Fibronectin; while in wound repair proinflammatory cytokines, such as IL-6 and IL-8, which are produced in the ECM by fibroblasts and macrophages (Hoch, Schraufstätter, & Cochrane, 1996; Wijelath et al., 2006). Changing the ECM is a critical step of tumour progression. ECM-regulated signalling pathways can promote cancer cell migration and extravasation from the tumour, while the ECM component hyaluronan regulates blood vessel endothelium, a critical barrier for cancer cell metastasis. ECM components can also modulate the survival of cancer cells in circulation and at secondary sites.

The importance of ECM components in cancer is evidenced by studies showing that DCN mediates the inhibition of colorectal cancer growth in mice, while higher expression of SPARC has been shown to suppress colonic tumourigenesis in

mice and is associated with better disease-free survival (DFS) (Aoi et al., 2013; Bi et al., 2012; Chew et al., 2011). MMP7, which promotes the turnover of ECM, is a negative prognostic marker in CRC patients and has been shown to induce epithelial-to-mesenchymal transition (EMT) in other cancers (Klupp et al., 2016; Q. Zhang et al., 2017).

Because of the evidence linking these genes to CRC, I decided to look at the ECM components DCN, SPARC, and MMP7. In Figure 4.2.5A, the Log2 fold changes, taken from the RNA sequencing analysis, of these genes in the *Villin-Cre*^{ERT} *VANGL2*$^{flox/flox}$ mouse (versus the *WT*) were plotted. As described in figure 4.2.4, DCN and SPARC are both significantly downregulated in the *VANGL2*-deficient colonic epithelium compared to the *VANGL2* wild-type. In addition, MMP7 is significantly increased as opposed to the wild-type. I conducted validation of these three genes using qRT-PCR on *Villin-Cre*^{ERT} *VANGL2*$^{WT/WT}$ and *Villin-Cre*^{ERT} *VANGL2*$^{flox/flox}$ epithelium. We can see that DCN and SPARC are downregulated, while MMP7 is upregulated when *VANGL2* is lost (Figure 4.2.5B). However, we do see variable efficiency of *VANGL2* loss in the colonic epithelium, which gives variable relative expression in the *VANGL2*$^{flox/flox}$ genotype, potentially highlighting the differences in efficiency between RNAseq and PCR.

Given the ECM components shown to be altered when *VANGL2* is knocked out, I decided to assess a further 5 genes which have been shown to be important regulators of the ECM. Epithelial tissue from both genotypes was assessed via qRT-PCR for transcript expression of MMP2, MMP9, αSMA (α-smooth muscle actin), TIMP1 (Tissue inhibitor of Metalloproteinases 1) and TIMP3 (Figure 4.2.5C). MMP2 and MMP9, like MMP7, are members of the matrix metalloproteinase family of genes (although each member differs in its substrate) and are involved in the turnover of the ECM (Said, Raufman, & Xie, 2014). αSMA is a member of the actin family of proteins, and is a marker of mesenchyme (relevant to EMT indication) (X. Y. Han et al., 2013). TIMPs promote ECM stability by inhibition of MMP activity, and while TIMP3 has been shown to suppress malignant behaviour of CRC cells, increased TIMP1 expression is associated with progression and metastasis in human CRC, presenting a mixed picture of TIMPs as a prognostic marker in CRC (H. Lin et al., 2012; Song et al., 2016). In the *Villin-Cre*^{ERT} *VANGL2*$^{flox/flox}$ epithelium, MMP2 is downregulated by 38%. MMP9 expression in the *VANGL2*-deficient cells is reduced by 53%, but this does not reach statistical significance. This is likely due to the large variability in relative expression in the WT samples (n = 3), and a larger dataset may find significantly different expression. In PCR analysis, relative expression of αSMA is increased by 142% by the loss of *VANGL2*. TIMP1 expression is reduced by 53%, whereas TIMP3 expression does not change between genotypes.

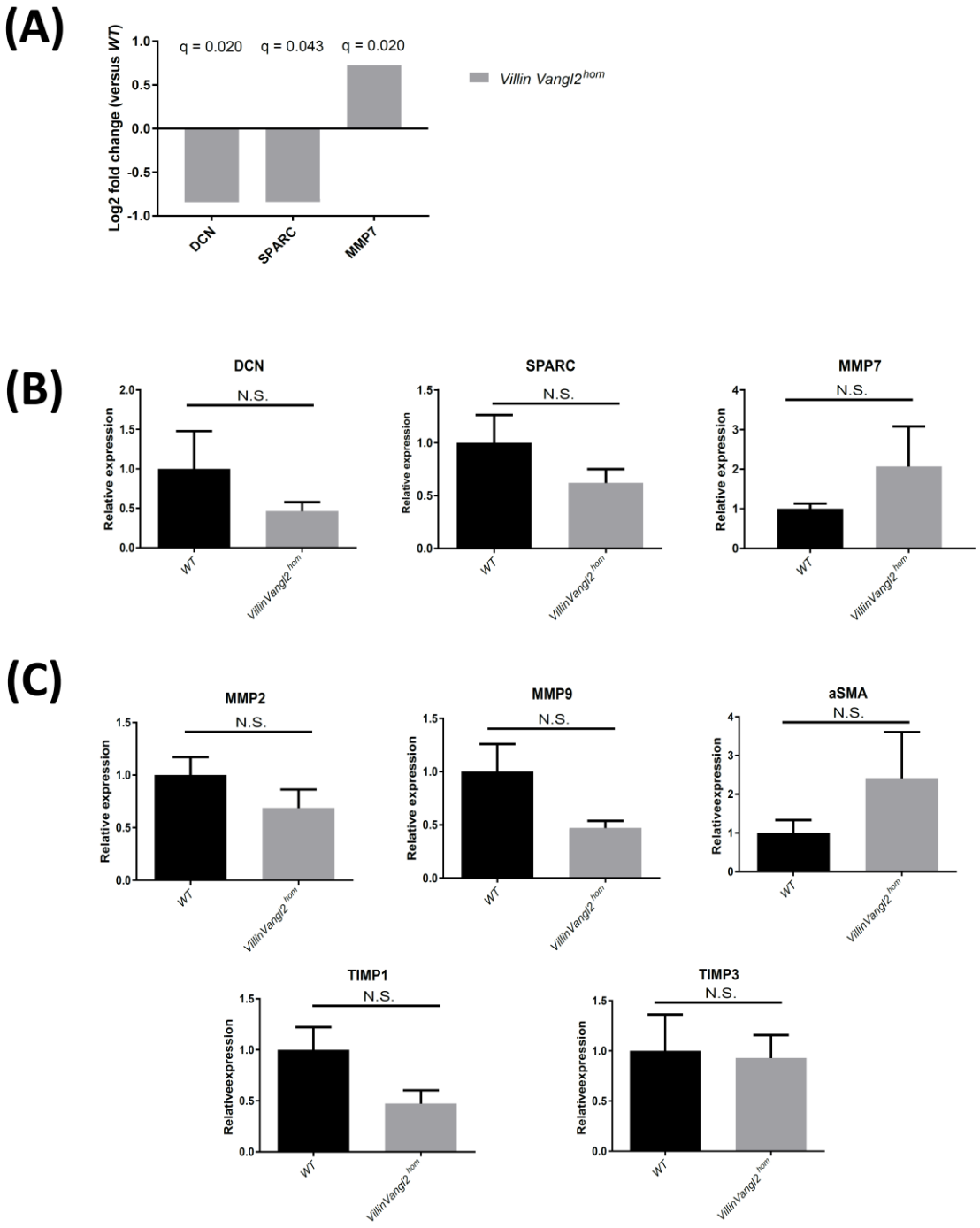


Figure 4.2.5 Investigation of VANGL2-mediated regulation of the ECM

(A) RNA Sequencing Log2 fold changes of ECM components DCN, SPARC, and MMP7 in *Villin Vangl2*^{hom} (versus *WT*) **(B)** qRT-PCR validation of RNA sequencing data in (A). Epithelial tissue from indicated genotypes was obtained and RNA extracted. cDNA was synthesised and qRT-PCR was then performed for each of DCN, SPARC, and MMP7. **(C)** qRT-PCR targeting other ECM components including MMP2, MMP9, aSMA, TIMP1, and TIMP3. For each gene, expression values are relative to the WT. All expression normalised to Ppia. Mean (SD) values shown. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

4.2.6 Histological investigation of *VANGL2*-mediated regulation of the ECM

Transcriptional changes in extracellular matrix (ECM) components in the colonic epithelium, as a result of the loss of *VANGL2*, does not necessarily mean that the composition of the ECM is changes, so I decided to assess ECM protein quantity in *VANGL2*flox mice and control. Fixed colon from *WT* and *Villin-Cre*^{ERT} *VANGL2*$^{flox/flox}$ was stained for the ECM components DCN, SPARC, and COL1 (type I collagen) (Figure 4.2.6A). Type I collagen is the most abundant collagen within the ECM and is therefore a good indicator of interstitial integrity of the colon. Staining of each protein was quantified computationally by identifying positive areas of staining and stratifying staining into low, moderate, and high expression. Protein quantity is calculated as a 'quickscore' (QS), which is a function of staining area and staining intensity (Figure 4.2.6B).

DCN is expressed highly within the colonic epithelium, while SPARC has a lower level of expression. *Villin-Cre*^{ERT} *VANGL2*$^{flox/flox}$ colons have unchanged expression of both these markers when compared to the *WT* suggesting that there is either a lack of concordance between transcript and protein or that the half-lives of these ECM proteins is very stable and therefore changes are not seen in the time

course examined. In these genotypes, COL1 protein is significantly decreased in the *Villin-Cre^{ERT} VANGL2$^{flox/flox}$* mouse when compared to the *WT* (Mean QS of 8.68 and 13.84, respectively).

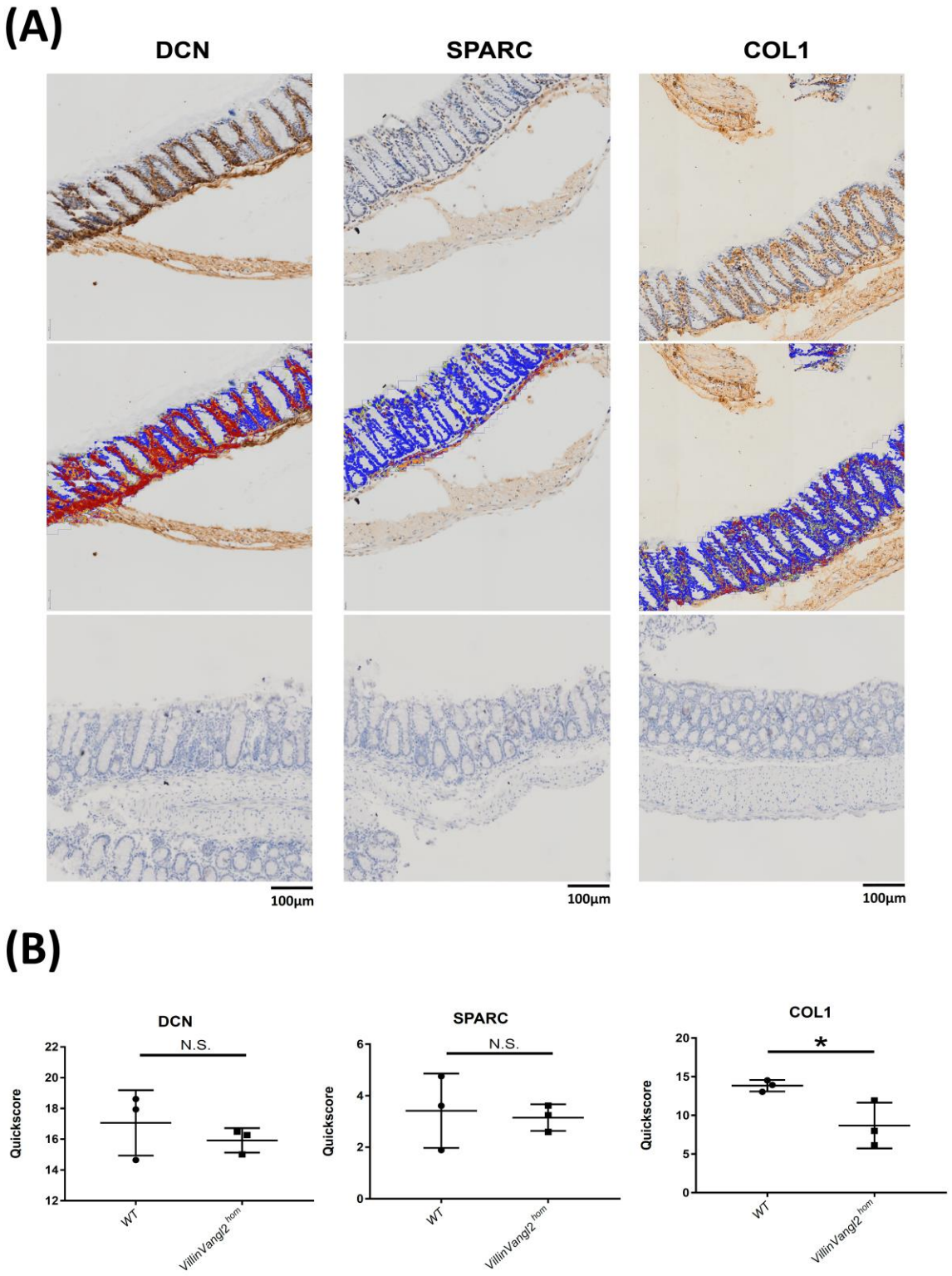


Figure 4.2.6 Histological investigation of VANGL2-mediated regulation of the ECM

(A) Fixed colon from *WT* and *Villin Vangl2*^{hom} mice was stained for ECM components DCN, SPARC, or COL1 and visualised using DAB. Representative image of *WT* shown above annotated image of cellular analysis (centre). Bottom row depicts negative controls (stains minus primary antibody). Nuclei are identified in blue, while DAB intensity of protein is categorised into low (yellow), moderate (orange), and high (red) areas of expression. **(B)** Quantification of ECM component expression from *WT* and V*illin Vangl2*^{hom} mouse colonic epithelium. Analysis was isolated to colonic epithelium. Quickscore = staining intensity x % area stained. Mean (SD); students t-test. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

4.2.7 Investigation of ECM-regulating pathways in the *Villin-Cre^{ERT} VANGL2$^{flox/flox}$* mouse

Given the transcriptional changes in extracellular matrix (ECM) components we see in the loss of *VANGL2* in colonic epithelial cells, as well as the decrease in type I collagen we see immunohistochemically, I assayed transcriptional targets of three different signalling pathways linked to ECM regulation.

It is known that Wnt/PCP signalling leads to activation of c-Jun via c-Jun N-terminal Kinase (JNK) (Boutros, Paricio, Strutt, & Mlodzik, 1998; Yamanaka et al., 2002). C-Jun forms a heterodimer with c-Fos to form the AP-1 (Activator protein-1) transcription factor. Research has shown that type I collagen can be regulated by the AP-1 transcription factor (TF) in osteoblasts, and that in cardiac ECM remodelling c-Jun suppresses the expression of ECM proteins, including type I collagen (other proteins suppressed include type III collagen and Fibronectin (FN)) (Windak et al., 2013; C. C. Wu et al., 2006). Other research shows that the AP-1 and c-Fos mediate MMP transcription (Bergman et al., 2003; S. Han, Ritzenthaler, Sitaraman, & Roman, 2006). Again, transcript analysis was performed via qRT-PCR and it was found that mRNA levels of cJun and Fos1 are suppressed in the *Villin-Cre^{ERT} VANGL2$^{flox/flox}$*

epithelium compared to the *WT* (Figure 4.2.7A), however the data does not meet statistical significance.

The Protein kinase C (PKC) family is a key signalling target of the non-canonical Wnt/Ca^{2+} pathway (Luna-Ulloa, Hernández-Maqueda, Castañeda-Patlán, & Robles-Flores, 2011). In a mouse model of muscular dystrophy, it was found that ablation of PKCθ reduced the expression of MMP9, a known promoter of fibrosis (e.g. type I collagen deposition) (Madaro et al., 2012). I found that loss of *VANGL2* in the mouse colonic epithelium leads to a significant reduction of PKCθ expression (Figure 4.2.7B). As earlier I found that MMP9 and type I Collagen expression is reduced when *VANGL2* is lost, this would suggest that loss of *VANGL2* leads to MMP9-mediated type I Collagen deposition, and that this is mediated through PKCθ. This could be tested through the use of PKCθ loss-of-function constructs, as has been used in breast cancer both *in vivo* and *in vitro*, to evaluate collagen deposition in the *VANGL2*-deficient colon (Byerly, Halstead-Nussloch, Ito, Katsyv, & Irie, 2016). Opposing this theory, Paoletti et al showed that PKCθ has been shown to be crucial for maintaining normal cardiac ECM homeostasis, and that ablation of the gene leads to an increase in fibrosis, including type I collagen (Paoletti et al., 2010). Therefore, PKCθ may have context-dependent roles in fibrosis.

One of the most important pathways regulating fibrosis and the ECM is Hippo signalling. This pathway, often transduced by the effector molecules yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), can promote fibrosis (F. Liu et al., 2015). This is particularly interesting given *VANGL2* carries a c-terminal PDZ-binding domain. Inhibition of YAP/TAZ signalling has been shown to attenuate expression of type I collagen in lung fibroblasts. Interestingly, research has shown that Wnts can activate YAP/TAZ through a non-canonical Wnt pathway (H. W. Park et al., 2015). I conducted qRT-PCR looking at 4 target genes of YAP/TAZ signalling: WNT5B, BMP4, CYR61, and IGFPB1 (Figure 4.2.7C). I found that loss of *VANGL2* had no effect on the YAP/TAZ pathway target genes assessed.

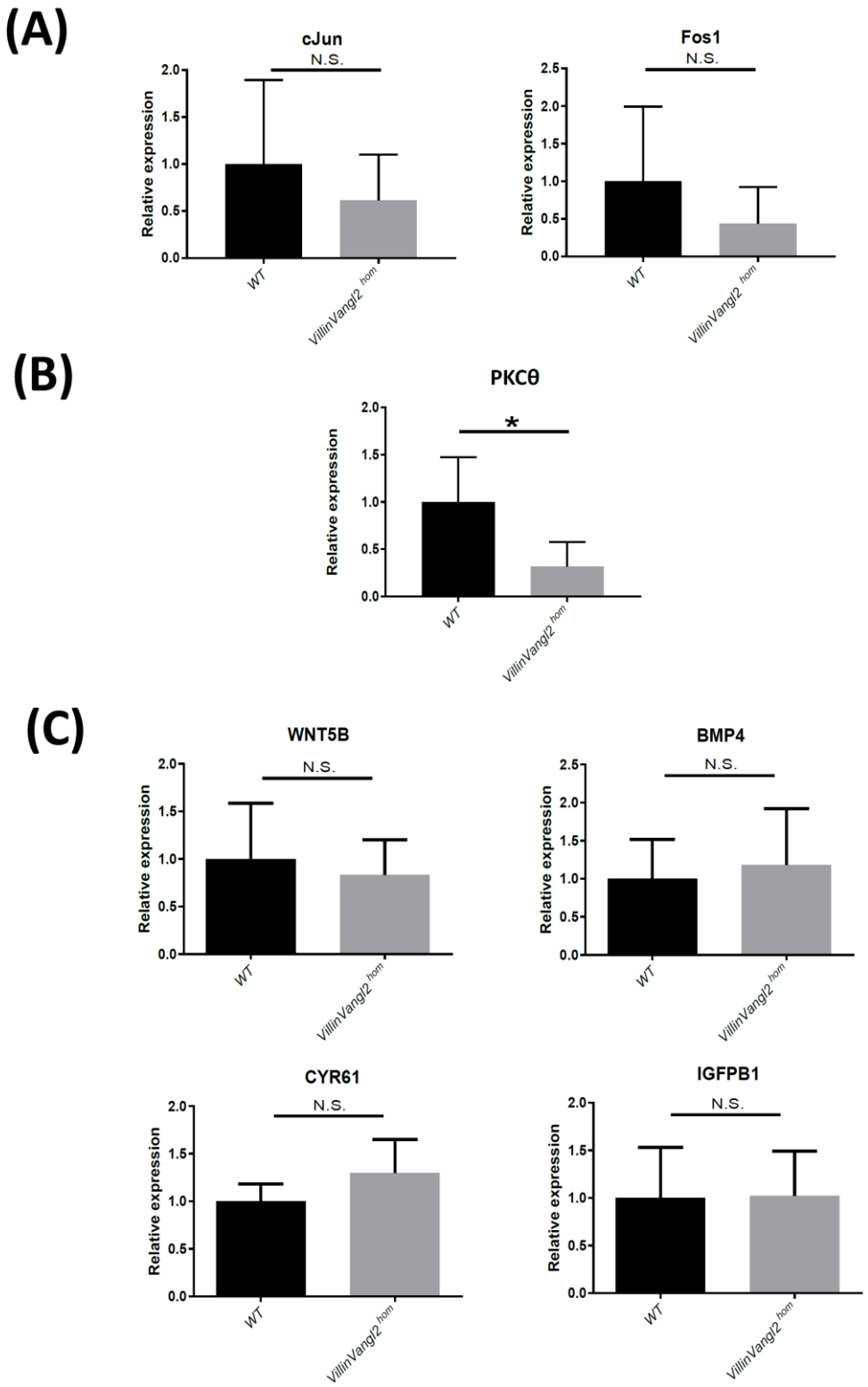


Figure 4.2.7 Investigation of ECM-regulating pathways mediated by VANGL2

Expression analysis of target genes of **(A)** Wnt/PCP signalling, **(B)** Wnt/Ca^{2+} signalling, and **(C)** YAP/TAZ (Hippo) signalling. Epithelial tissue from indicated genotypes was obtained and RNA extracted. cDNA was synthesised and qRT-PCR was then performed for each of the indicated genes. For each gene, expression values are relative to the *WT*. All expression normalised to Ppia. Mean (SD) values shown. Students t-test used. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. $N = 4$.

4.3 Discussion

Knockout of *VANGL2* in the mouse adult intestinal epithelium did not affect the morphology of the tissue in an H&E stain. This could be expected as previous conditional knockout studies did not find morphogenic changes in tissue but rather changes in cell migration/wound healing (Findlay et al., 2016). In future studies, cell migration assays could be carried out *in vitro* with VANGL2-deficient colorectal cell lines. 'Scratch' or 'wound healing' assays can be employed to quantify the cellular migration/wound healing ability of cells (Justus, Leffler, Ruiz-Echevarria, & Yang, 2014).

It has been indicated that, in CRC cells, *VANGL2* constricts proliferation in cells (Piazzi et al., 2013). However, in breast cancer cells *VANGL2* promotes proliferation, suggesting that *VANGL2* supports proliferation (Puvirajesinghe et al., 2016). I found that loss of *VANGL2* in the colonic epithelium has no effect on proliferation. Overall, it seems that *VANGL2*'s role in cellular proliferation acts on a context-dependent basis. For example, *VANGL2* function may be affected by the presence of an oncogene (such as oncogenic mutations in APC or K-RAS), whereby *VANGL2* might modify proliferative ability in the context of a tumour.

Canonical Wnt signalling target expression is not regulated by *VANGL2* in colonic epithelial cells. Previous work has shown that *VANGL2* can act to activate canonical Wnt signalling, or to inhibit it (Dyberg et al., 2016; Shafer et al., 2011). While my data indicates that *VANGL2* does not affect canonical Wnt signalling, it is important to note that many of these pathways are regulated at the protein level, and so we may not see signalling changes through transcriptional analysis. Further work examining protein expression (for example, in using western blotting) of WNT/β-catenin signalling markers is necessary to define the canonical Wnt signalling landscape in our model.

I find several indications of perturbation of downstream Wnt/PCP signalling in our analysis in the *VANGL2* LOF epithelium, including downregulation of AP-1 subunit transcription. FRA1 has been shown to be a regulator of epithelial-to-mesenchymal transition (EMT) in CRC cells, and is found at the invasive front in human colorectal cancers (Diesch et al., 2014). This suggests that FRA1/FRA2-dependent AP-1 activity is abrogated when *VANGL2* is lost in the colonic epithelium. This loss of activity may have a suppressive effect on EMT and would, therefore, be protective in CRC. Suppression of EMT can be quantified via examining expression of

the epithelial marker E-Cadherin, either at the transcriptional or protein level (Zeisberg & Neilson, 2009). If EMT is suppressed in our VANGL2-deficient epithelial cells, we would see increased levels of E-Cadherin over our WT cells. Interestingly, there is evidence that cell-ECM interactions can stimulate AP-1 activity, suggesting an AP-1-ECM signalling feedback loop (Troussard, Tan, Yoganathan, & Dedhar, 1999). I also show that members of the RhoA signalling family are upregulated when *VANGL2* is lost. Members of this pathway, including ROCK1 and ROCK2, and been shown to have oncogenic roles in CRC (Qiu et al., 2015; Sari et al., 2013; G. Zhang, Yang, & Chen, 2016). Taken together, these data suggest that *VANGL2* may be suppressing RhoA/ROCK signalling in the intestinal epithelium. While previous evidence has shown that loss of functional *VANGL2* in development disrupts RhoA and ROCK1 localisation during development, there is no evidence that overall expression is altered (Phillips et al., 2005). *VANGL2* may, in fact, act to control localisation of RhoA/ROCK1 expression in development and continue to do so in the colonic epithelium.

My transcriptional analysis on *VANGL2* LOF epithelial cells show downregulation of ECM components DCN and SPARC, as well as the upregulation of matrix remodelling protein MMP7. Both DCN and SPARC have been implicated as a tumour suppressors in CRC (Chew et al., 2011; Mlakar et al., 2009). Higher MMP7

expression has been shown to predict poor overall survival (OS) and disease-free survival (DFS) in CRC (Sun, Zhang, Qi, Zhou, & Lv, 2015).

Further analyses carried out found MMP2 and MMP9 to be downregulated when *VANGL2* is lost. These comprise the gelatinase sub-family of MMPs, and increased expression of these correlate with worse CRC outcome (Said et al., 2014). Mesenchymal marker αSMA is upregulated when *VANGL2* is lost. Increased expression of this marker in epithelial cells is an EMT-like phenotype (Valcz et al., 2012). Interestingly, inhibition of ROCK has been shown to impede EMT in the human cornea. This suggests that ROCK signalling promotes EMT (Q. Wu, Ouyang, Xie, Ling, & Huang, 2017). My data supports this theory, as in the *Villin-Cre^{ERT} VANGL2$^{flox/flox}$* mouse we see increased ROCK expression concurrent with increased expression of αSMA. TIMP-1 is found to downregulated, which, despite being a known inhibitor of MMP9, is also associated with poorer prognosis in CRC (Jensen, Vainer, Bartels, Brünner, & Sørensen, 2010). The role of ECM regulators in this context is complex with regards to CRC and further work is necessary. For example, gelatin zymography can be utilised to quantify MMP2 and MMP9 activity (as these are gelatinase enzymes) (Toth, Sohail, & Fridman, 2012).

In concordance with my transcriptional data, type I collagen is reduced in the colonic epithelium when *VANGL2* is lost. Interestingly, in a proteomic analysis type I Collagen was identified as being upregulated during tumourigenesis in CRC (Zou et al., 2013). It was also found that a type I collagen degradative telopeptide (CTx) correlated with tumour staging and worsened DFS. Therefore, *VANGL2* may help promote tumour initiation through increasing expression of type I Collagen.

The AP-1 transcription factor subunits and two of their targets, DCN and COL1A2, are upregulated by *VANGL2* in the epithelium. It has previously been shown that a proximal promoter region of the human DCN and COL1A2 genes are bound and activated by the AP-1 transcriptional complex (Chung, Agarwal, Uitto, & Mauviel, 1996; Mauviel et al., 1996). These data suggest that downstream Wnt/PCP signalling mediates ECM components through AP-1 activity, and that *VANGL2* promotes this activity.

The key downstream target of Wnt/Ca^{2+} signalling, PKCθ, is downregulated in the *VANGL2* LOF epithelium. While there are no known interactions between *VANGL2* and this non-canonical, Calcium dependant, Wnt signalling pathway, it is important to note that the non-canonical ligand Wnt5a can activate either Wnt/Ca^{2+} or Wnt/PCP signalling, and that as-yet-unknown cross-talk mechanisms may exist to

modulate each other. Similar to the Wnt/PCP pathway, Wnt/Ca^{2+} signalling has also been shown to be essential for convergent extension during embryogenesis, as well as in polarised cell migration (Kinoshita, Iioka, Miyakoshi, & Ueno, 2003; Minami, Oishi, Endo, & Nishita, 2010), indicating again that these pathways could be integrated.

Chapter 5: *VANGL2*-mediated signalling enhances tumourigenesis and regulates the ECM in intestinal cancer

5.1 Introduction

The data discussed in chapter 4 focussed on the identification of pathways mediated by *VANGL2* in the adult colon. This revealed that *VANGL2* potentially regulates the extracellular matrix (ECM), and further analysis showed that this might be because of downstream non-canonical Wnt signalling. This is in addition to chapter 3 revealing that overexpression of *VANGL2* in human colorectal cancer (CRC) results in worsened disease-free survival (DFS). In this chapter I will be exploring how *VANGL2* mediates cancer development, and examining whether these ECM alterations play a part in this.

As previously discussed, *VANGL2* upregulation is found in multiple human carcinomas (cancers of the epithelium) (Hatakeyama et al., 2014). As well as this, *VANGL2* promoter methylation is associated with increased tumour grade and BRAF mutations in CRC (both of which are associated with poorer survival) (Piazzi et al.,

2013). The evidence around *VANGL2*s role in CRC (i.e. as a tumour suppressor or as an oncogene) is therefore conflicting. Interestingly, *VANGL2* has previously been shown to have a role in ECM remodelling in cancer (Cantrell & Jessen, 2010; B. Blairanne Williams et al., 2012). Here, *VANGL2* loss in fibrosarcoma cancer cells leads to MMP14-dependent ECM invasion and directed migration. *VANGL2* knockdown cells show increased activation of MMP2, higher membrane localisation of MMP14 (a membrane tethered MMP) and decrease in ECM component fibronectin, showing that *VANGL2* acts to restrict MMP-mediated cleavage of ECM proteins in these cells. This suggests that *VANGL2* restricts fibrosarcoma invasiveness *via* ECM turnover.

Remodelling of the ECM is a well-known and necessary step in colorectal cancer progression. The ECM is composed of the basement membrane (BM), which surrounds epithelial cells and acts as a barrier and a regulator of epithelial function; and the stromal matrix, which contains a multitude of molecules, which form the bulk of the mesenchyme in the intestine. Found in the BM, laminins are found to be lost in colon cancer cells and are a transcriptional target of the tumour suppressor Smad4 (loss of which is associated with malignancy) (Zapatka et al., 2007). Type I collagen (COL1) is highly expressed at the invasive front of human CRC. Research has shown that type I collagen promotes a stem-like phenotype (indicated by Bmi1 and CD133) in human CRC cells (Kirkland, 2009). Fibronectins (such as FN1) are glycoproteins within the ECM and are important for cellular migration through fibronectin-integrin

interactions. As such, colon cancer cell stimulation with fibronectin has been shown to increase migration and invasiveness of cancer cells, and patients with high expressing FN1 CRC have poorer prognosis (Ding, Li, Wang, Wang, & Wu, 2008; Yi, Xiao, Ding, Luo, & Yang, 2016). Another contributor to the ECM, proteoglycans, are also implicated in CRC development. Loss of syndecan-1 is associated with TNM stage and with metastasis (Hashimoto, Skacel, & Adams, 2008). Decorin is another proteoglycan which is present in the stroma and binds to type I collagen. It has been shown to inhibit CRC growth and cancer cell migration via interaction and stabilisation of E-cadherin (Bi et al., 2012). In other cancers, Decorin has been linked to tumour development, progression, and angiogenesis (Bi et al., 2008; Davies et al., 2001; Fiedler et al., 2008; Santra, Eichstetter, & Iozzo, 2000). Decorin can promote angiogenesis in osteosarcoma through facilitating endothelial cell adhesion and migration on type I collagen. This is mediated through binding to α2β1 integrin and promoting integrin-collagen interaction. Integrins are cellular receptors for ECM molecules (such as fibronectin, laminin, and collagen) and it has been demonstrated that poorly differentiated cancers are characterised by high amounts of integrin-ECM interactions. Furthermore, activation of integrins with collagen in CRC cells activates COX-2 signalling, and contributes to cell migration (Broom, Massoumi, & Sjölander, 2006; Murillo, Rychahou, & Evers, 2004).

Conversely, the ECM needs to be turned over and remodelled as well as being deposited. Metalloproteases (or MMPs) are the principal ECM degrading enzymes and therefore play a key role in ECM turnover (a crucial step in cancer growth, invasion, and metastasis). MMP1, -2, -3, -7, -9, -13, and -14 (also known as MT1-MMP) are all known to be elevated in human CRC (Said et al., 2014). MMP2 and MMP9 constitute the gelatinase sub-family of MMPs. These can be secreted by stroma upon induction from CRC cells, and increased levels of MMP9 is associated with malignancy (Mook, Frederiks, & Van Noorden, 2004). Tissue inhibitors of metalloproteinases (TIMPs) are also involved in CRC development. Surprisingly, increased plasma levels of TIMP-1 in CRC patients has been shown to be associated with worsened overall survival (OS) and time to progression (TTP) of the disease (Sørensen et al., 2007). This surprising paradox between MMPs and TIMPs in CRC could be a result of cancer stage or cancer subtype-dependent effects of either protein.

As *VANGL2* has an unknown role in CRC, it could potentially regulate the ECM. I set out to test the hypothesis that *VANGL2* is involved in mediating CRC development via ECM regulation.

5.2 Results

5.2.1 Long-term epithelial loss of *VANGL2* is not sufficient for intestinal tumourigenesis

It is unclear if loss of Vangl2 itself is oncogenic. Therefore, I carried out an experiment to find if loss of *VANGL2* alone in the healthy epithelium is sufficient for intestinal tumourigenesis. *Villin-Cre^{ERT} VANGL2$^{WT/WT}$*, *Villin-Cre^{ERT} VANGL2$^{Flox/WT}$*, and *Villin-Cre^{ERT} VANGL2$^{Flox/Flox}$* mice (at ages between 8-12 weeks old, to allow for a fully developed adult intestinal tract) were induced with IP injections of tamoxifen aged for up to 6 months, before being culled (Figure 5.2.1A). Over this period, survival of each genotype was compared to the *VillinCre^{ERT} APC$^{flox/WT}$* cancer model. *VANGL2* flox genotypes had 100% survival over this period, while the cancer model resulted in only 53% survival (Figure 5.2.1B). This suggests that long-term mucosal loss of *VANGL2* does not affect survival. Intestines from the cancer model and each of the *VANGL2^{flox}* genotypes were assessed for intestinal tumour number. *APC$^{flox/WT}$* mice had an average of 45 tumours, while heterozygous and homozygous loss of *VANGL2* resulted in no observed tumours (Figure 5.2.1C). Intestines between *Villin-Cre^{ERT} VANGL2$^{WT/WT}$*, *Villin-Cre^{ERT} VANGL2$^{Flox/Flox}$*, and *Villin-Cre^{ERT} VANGL2$^{Flox/Flox}$* mice were compared histologically, and all were found to be similar and healthy (Figure 5.2.1D). This evidence shows that loss of *VANGL2* alone is not sufficient for tumourigenesis.

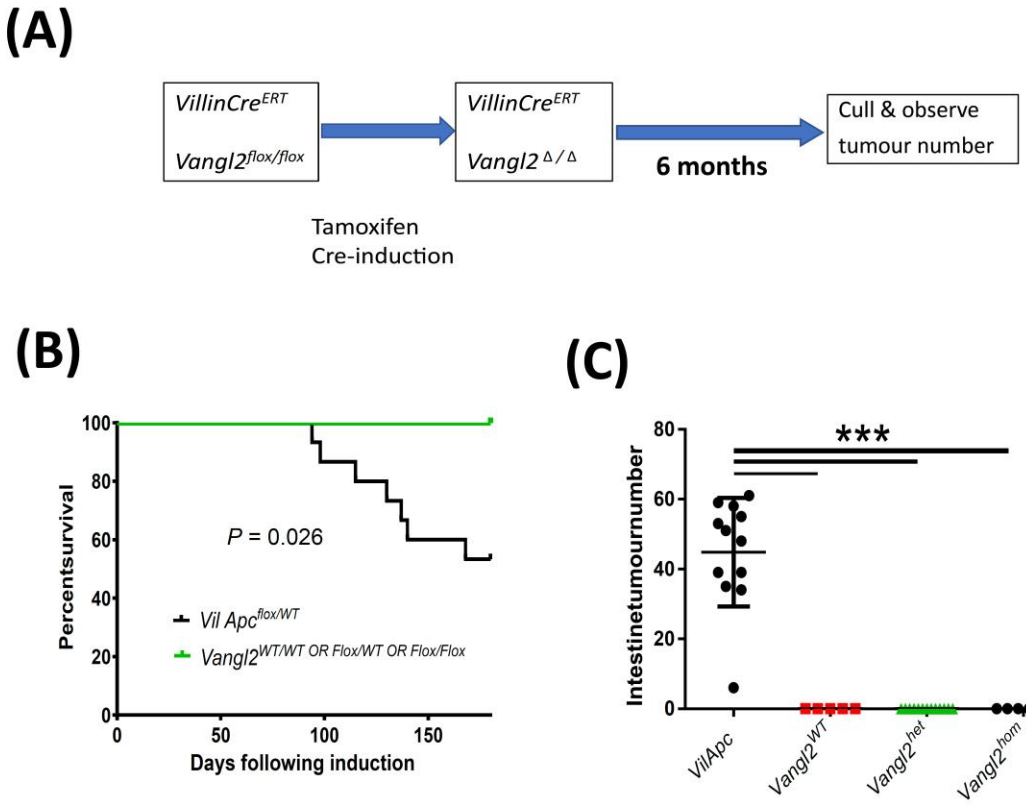


Figure 5.2.1 Loss of VANGL2 is not sufficient for intestinal tumourigenesis in mice

(A) Schematic of experimental strategy for long term-loss of VANGL2. *VillinCre*^{ERT} *Vangl2*^{flox} mice are induced with intraperitoneal injections of tamoxifen. Six months following induction mice are culled and intestines assessed for tumour number. **(B)** Kaplan-Meier survival plot of *VillinCre*^{ERT} *Vangl2*$^{flox/flox}$, *Vangl2*$^{flox/WT}$, and *Vangl2*$^{flox/flox}$ mice compared to *VillinCre*^{ERT} *APC*$^{flox/WT}$ mice over 6 months. 100% survival was recorded in VANGL2 loss alone was recorded over 180 days, compared to 53% survival in *VillinCre*^{ERT} *APC*$^{flox/WT}$ mice. Log-rank test (p = 0.0011). **(C)** Cumulative tumour number in the intestine between genotypes, counted macroscopically. No polyps were found in any of the *Vangl2*^{flox} genotypes. Mean (SD); one way ANOVA. N.S. (not significant) = p > 0.05 * = p < 0.05, ** = p < 0.01, *** = p < 0.001. **(D)** Intestines with long term loss of VANGL2 are histologically similar to *Vangl2*^{WT} and do not contain tumours. Representative microscopy images of fixed murine intestines of *VillinCre*^{ERT}*Vangl2*$^{WT/WT}$, *Vangl2*$^{flox/WT}$, and *Vangl2*$^{flox/flox}$ mice. N = 6 per genotype.

5.2.2 Intestinal inactivation of *VANGL2* in the *VillinCre^{ERT} APC$^{flox/WT}$* mouse does not alter survival

To investigate how *VANGL2* might mediate colorectal cancer development, I decided to look at the loss of the gene in an intestinal tumourigenesis model. *APC$^{flox/WT}$* mice lose a functional copy (through creation of a frameshift mutation at AA580) of APC when recombination is induced by *Cre* (Colnot et al., 2004). Compared to *APC^{Min}* mutant, mice heterozygous for functional APC are prone to a large number of polyps in the small intestine, and a small amount in the colon, with which usually results in early mortality due to anaemia (Johnson & Fleet, 2013). Spontaneous loss of heterozygosity by mutation of the remaining *APC^{WT}* allele causes tumour initiation in this model, which leads to constitutive WNT/β-catenin signalling and uncontrolled proliferation.

By crossing *APC$^{flox/WT}$* mice with our *VANGL2* deletion model used in chapter 4, I created the *Villin-Cre^{ERT} APC$^{flox/WT}$ VANGL2$^{flox/flox}$* model. This allowed me to induce intestinal epithelial-specific loss-of-function (LOF) of *VANGL2* to assess its effect on intestinal tumourigenesis in the mouse. *APC$^{flox/WT}$* mice had loxP sites targeted to each side of *APC* exon 14 that was lost upon induction with *Cre* (Figure 5.2.2A). I induced *VillinCre^{ERT}* expression via intraperitoneal injection of tamoxifen, which led

to subsequent intestinal tumourigenesis (Figure 5.2.2B). All three genotypes (*VillinCre^{ERT} APC$^{flox/WT}$ VANGL2$^{WT/WT}$* **or** *VANGL2$^{Flox/WT}$* **or** *VANGL2$^{Flox/Flox}$*) were initially healthy, and experienced symptoms from intestinal tumourigenesis from around 50 days onwards. Symptoms (which progressed over the duration of the experiment) include hunching, rectal bleeding, and anaemia. These expected symptoms were seen in all genotypes. Mice were sacrifice when scored disease severity reached endpoint status, typified by pale feet (caused by anaemia) and hunching (up to 300 days following induction). Survival between genotypes was not significantly different (Figure 5.2.2C). The median lifespan of *VANGL2$^{WT/WT}$* mice was 182 days, while *VANGL2$^{Flox/WT}$* was 168 days, and *VANGL2$^{Flox/Flox}$* was 167 days (Log-rank p = 0.8723). Therefore, partial, or full loss of functioning *VANGL2* in the intestine does not alter the survival rate of *VillinCre^{ERT} APC$^{flox/WT}$* mice following intiation of CRC.

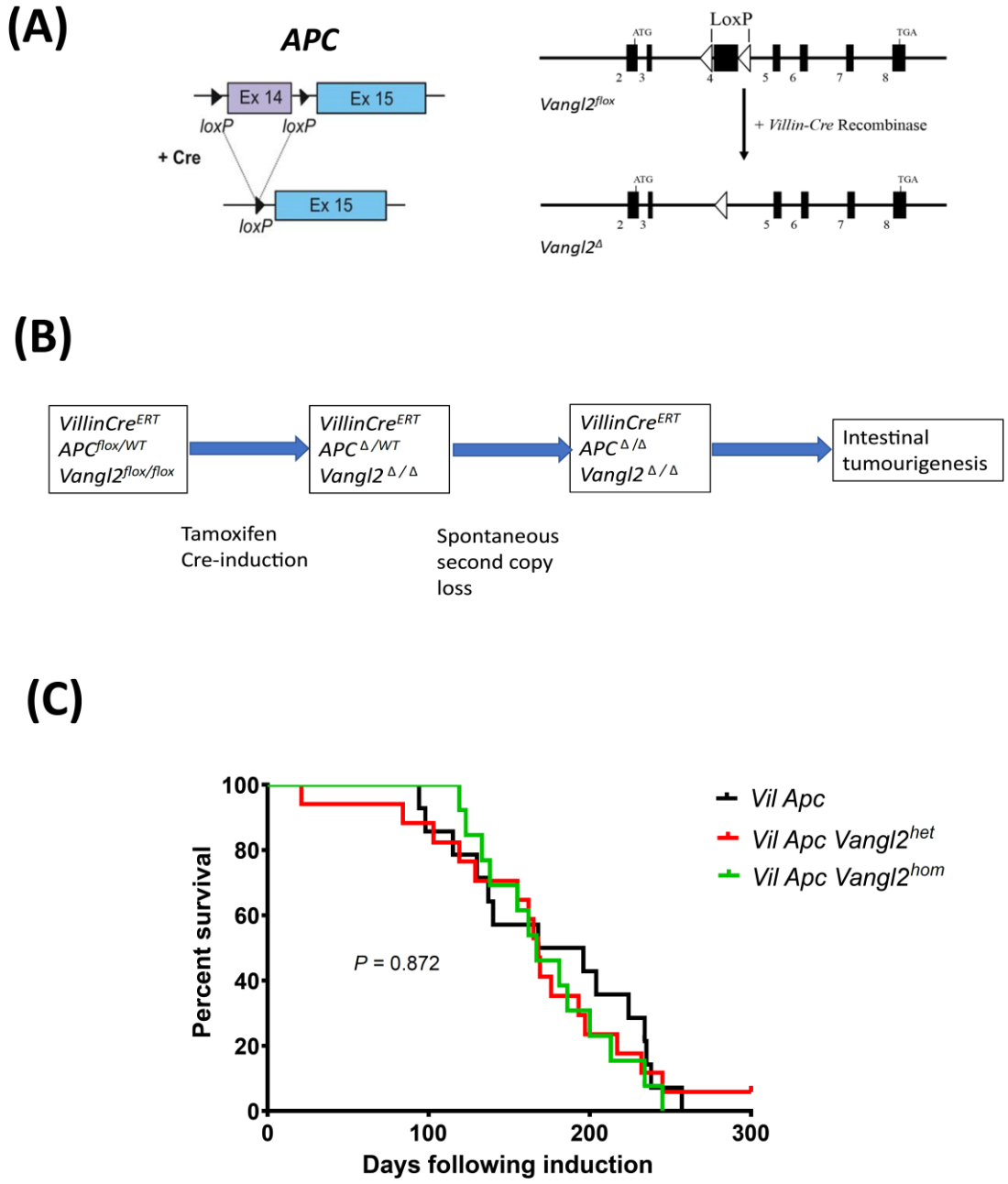


Figure 5.2.2 Intestinal inactivation of VANGL2 in the *VillinCre^{ERT} APC$^{flox/WT}$* mouse does not alter survival

(A) Schematic of exons 14-15 of the mouse *APC^{flox}* allele, and resulting exon 14 deletion induced by *Cre* recombinase. Also shown is the diagram outlining Cre-induced recombination of VANGL2 exon 4 in the mouse intestinal epithelium used in chapter 4. **(B)** Experimental strategy for VANGL2 deletion in APC-deficient intestinal tumour initiation. *VillinCre^{ERT} APC$^{flox/WT}$ Vangl2$^{WT/WT\ or\ Flox/WT\ or\ Flox/Flox}$* mice were given intraperitoneal injections of tamoxifen to induce intestinal epithelial specific loss of APC and VANGL2. Mouse intestine is then predisposed to tumour formation *via* spontaneous second copy loss of APC. **(C)** Kaplan-Meier survival plot of *VillinCre^{ERT} APC$^{flox/WT}$ Vangl2$^{WT/WT\ or\ Flox/WT\ or\ Flox/Flox}$* mice, up to 300 days following induction. There was no significant difference in survival in either partial (median survival = 168) or full loss (167 days) of VANGL2 when compared to VANGL2$^{WT/WT}$ (182 days). Log-rank test (P = 0.8723). *N* = 12 per genotype.

5.2.3 Loss of *VANGL2* ameliorates tumour formation in inactivated APC-driven intestinal tumourigenesis

Tumours were examined microscopically and were deemed to be histologically similar across genotypes, in tumour differentiation and grade (Figure 5.2.3A). Upon inspection by a histopathologist, tumours from both genotypes were classified as tubular adenomas with low grade dysplasia, with no evidence of invasion. No evidence of mucosal invasion or metastasis was found with mice up to 300 days following induction.

Tumour number within the small intestine and colon of each genotype was quantified (Figure 5.2.3B). All visible polyps were included in counts (with small and large polyps being counted equally), and tumour diameter was measured in mm. Changes in number of tumours can indicate an alteration in tumour initiation rates. $APC^{flox/WT}$ $VANGL2^{Flox/Flox}$ mice developed significantly fewer colonic tumours compared to both the $APC^{flox/WT}$ $VANGL2^{Flox/WT}$ and the $APC^{flox/WT}$ (average of 9.2 tumours versus 16.5 and 17.1, $p < 0.05$). No change in number of colonic tumours was found between $APC^{flox/WT}$ and $APC^{flox/WT}$ $VANGL2^{Flox/WT}$ mice. Number of tumours was also assayed within the small intestine and across the whole intestine, and no significant alteration in tumour number was found when *VANGL2* is lost.

Next, I looked at colonic burden to assess total polyp growth between genotypes (Figure 5.2.3C). Tumour burden was calculated as the sum of all tumour diameters within the colon, small intestine, or whole intestine. Changes in this measure could indicate alterations in tumour initiation and/or growth. I found that $APC^{flox/WT}$ $VANGL2^{Flox/Flox}$ mice developed significantly less colonic tumour burden compared to both the $APC^{flox/WT}$ $VANGL2^{Flox/WT}$ and the $APC^{flox/WT}$ (average of 21.6 cm versus 40.6 and 41.8, $p < 0.05$). Again, no change in colonic tumour burden was found between $APC^{flox/WT}$ and $APC^{flox/WT}$ $VANGL2^{Flox/WT}$ mice, and no significant alteration of tumour burden was found in the small intestine or whole intestine, indicating that *VANGL2* plays a specific role in the formation of colonic tumours.

Finally, average tumour diameter was measured to assay tumour growth within each genotype (Figure 5.2.3D). Changes in this measure would indicate alteration to tumour growth rates within the intestine. I found that there was no alteration in average tumour diameter between genotypes in the colon, small intestine, or whole intestine. Therefore, as the homozygous loss of *VANGL2* results in an decrease in colonic tumour number, but not diameter, I can conclude that this is a tumour initiation phenotype and not tumour growth.

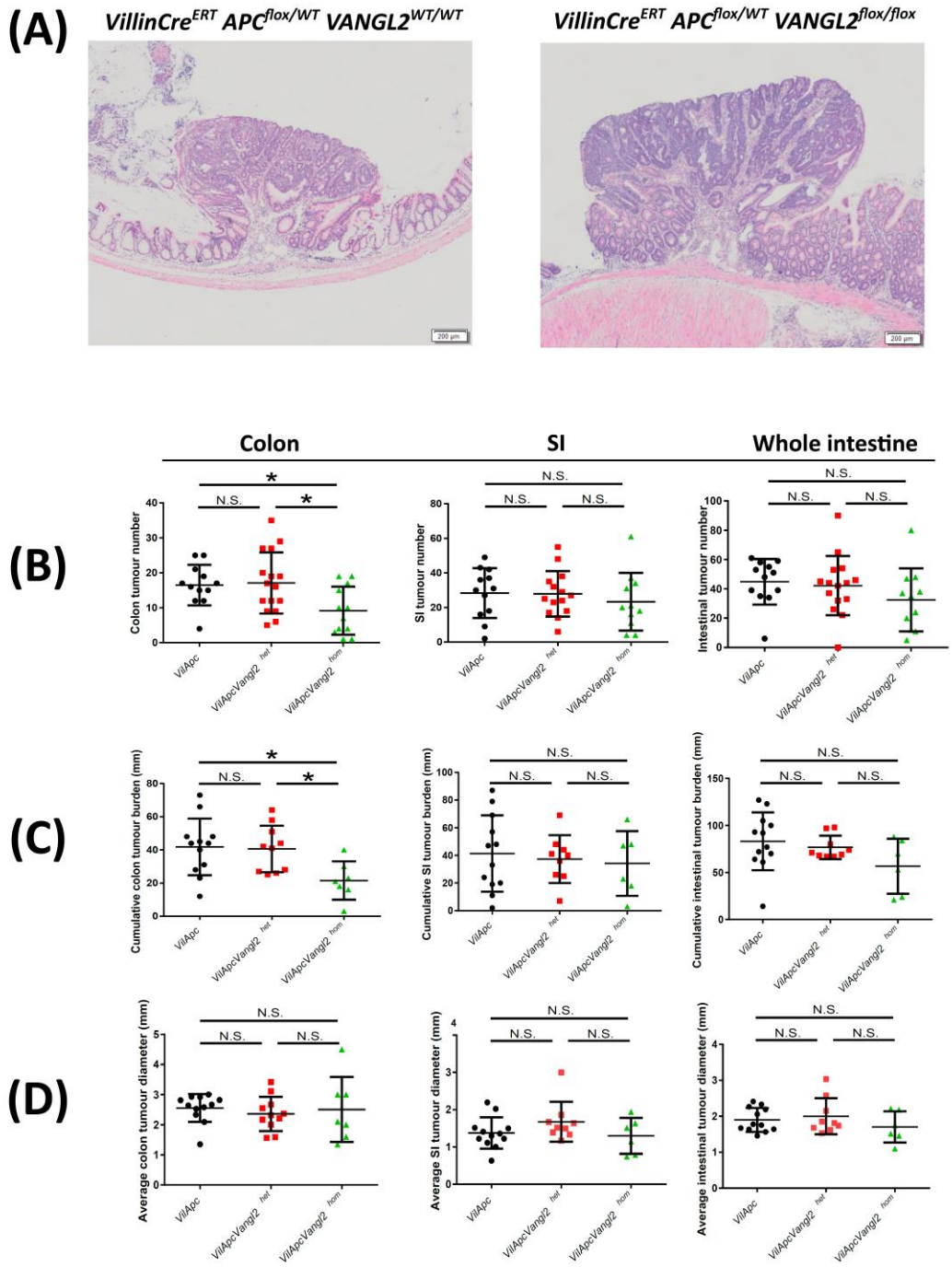


Figure 5.2.3 Loss of VANGL2 ameliorates tumour formation in APC-knockout driven intestinal tumourigenesis

(A) *Villin-Cre^{ERT} APC$^{Flox/WT}$ VANGL2$^{WT/WT}$* and *Villin-Cre^{ERT} APC$^{Flox/WT}$ VANGL2$^{Flox/Flox}$* colonic tumours are histologically similar. Sections from from each genotype were cut and H&E-stained. x2 magnification used. **(B)** Tumour numbers from each genotype were counted macroscopically in the colon, small intestine (SI), and the whole intestine. **(C)** Cumulative tumour burden between genotypes in the colon, SI, and the whole intestine. Tumour burden is represented by sum of all tumour diameters within each mouse. **(D)** Average tumour diameter between genotypes in the colon, SI, and the whole colon. Mean (SD); one way ANOVA. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. At least 6 mice per genotype were used in quantifications.

5.2.4 ECM component expression in *VANGL2*-deficient intestinal tumours

As we see reduced tumour formation in the colon *VANGL2* deletion, I decided to investigate cellular pathways which may be regulated by *VANGL2* to mediate tumour development. This was informed by my previous observation that in pre-malignant tissue *VANGL2* regulates the colonic ECM.

First, I looked at *VANGL2* expression in $APC^{flox/WT}$ and $APC^{flox/WT}$ $VANGL2^{Flox/Flox}$ tumours (Figure 5.2.4A). Tumours were isolated from the colon and qRT-PCR analysis performed. As expected, *VANGL2* expression is lost in the $VANGL2^{Flox/Flox}$ tumours. Relative expression was reduced to 2% of $VANGL2^{WT/WT}$ tumours, however this loss of transcript was not statistically significant due to variability in *VANGL2* expression in murine WT epithelium. Guided by the data found in chapter 4, I analysed ECM component expression within tumours from both genotypes (Figure 5.2.4B). I found an upregulation in Decorin, SPARC and TIMP1 mRNA expression in *VANGL2*-deficient tumours, while MMP9 expression is unchanged in wild-type tumours compared to those lacking *VANGL2*. Interestingly, I found in chapter 4 that each of these components are downregulated in *VANGL2*-deficient epithelium. It may be that in normal epithelial conditions the loss of *VANGL2* reduces ECM component deposition,

whereas in the highly altered state of the intestinal adenoma, cells may behave differently with the upregulation of the gene. Connective tissue growth factor (CTGF, or CCN2), is one of the most well documented proteins involved in ECM remodelling and colorectal cancer. In pancreatic cancer, upregulation has been found to correlate with early stages of tumour development, while upregulation has also been found to inhibit invasion and metastasis of CRC. Bennewith et al found that CTGF inhibition of xenografted CRC cells dramatically reduced tumour growth. This was found to be mediated by CTGF protection of CRC cells from hypoxia-mediated apoptosis (Bennewith et al., 2009). In a report from Lin et al, patients with stage II and stage III CRC with high CTGF tumour expression had both better overall and disease-free survival than patients with low CTGF expression (Been-Ren Lin et al., 2005). Reduction of CTGF levels in CRC cells implanted in BALB/c mice resulted in increased liver metastasis. In later work, it was revealed that lower CTGF levels in CRC patients was associated with higher peritoneal disease recurrence, and that CTGF decreases CRC cell adhesion ability in CRC cells (B.-R. Lin et al., 2011). Loss of *VANGL2* leads to an increase in expression levels of CTGF in tumours (Figure 5.2.4C). CTGF is a transcriptional target of Hippo (or YAP/TAZ) signalling and, interestingly, studies have shown that deregulation of Wnt/PCP signalling results in the activation of this pathway (Cordenonsi et al., 2011; Gujral et al., 2014).

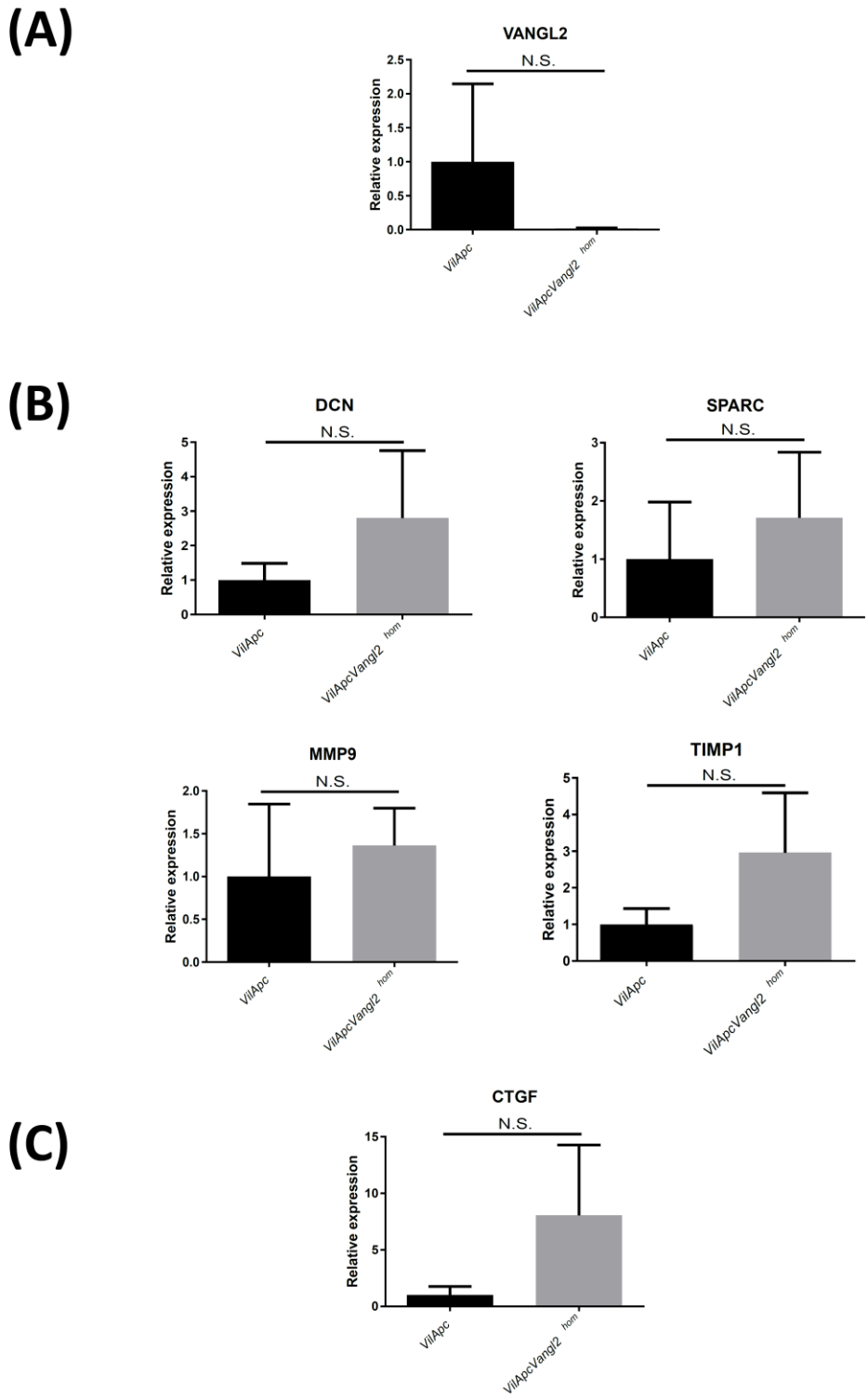


Figure 5.2.4 ECM component expression in VANGL2-deficient intestinal tumours

Tumours taken from *VillinCre*^{ERT} *APC*$^{flox/WT}$ *VANGL2*$^{WT/WT}$ and *VillinCre*^{ERT} *APC*$^{flox/WT}$ *Vangl2*$^{Flox/Flox}$ mice were used for qRT-PCR analysis of **(A)** VANGL2 , **(B)** ECM components DCN, SPARC, MMP9, TIMP1, and **(C)** CTGF expression. Expression presented relative to *VillinCre*^{ERT} *APC*$^{flox/WT}$ *VANGL2*$^{WT/WT}$ tumours. All expression normalised to Ppia. Student's t-test; Mean (SD) values shown. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. *N* = 3.

5.2.5 Immunohistological analysis of ECM-regulation by *VANGL2* within the colonic tumour environment

Given the upregulation of ECM transcripts observed in $APC^{flox/WT}$ $VANGL2^{Flox/Flox}$ tumours, I sought to look at the regulation of ECM components known to be important CRC histologically and determine whether these ECM proteins are differentially expressed in *VANGL2* WT vs floxed tumours.

Staining, using immunohistochemistry for the ECM components DCN, SPARC, type I Collagen (Col1), and Laminin was performed on fixed colon from $APC^{flox/WT}$ $VANGL2^{WT/WT}$ and $APC^{flox/WT}$ $VANGL2^{Flox/Flox}$ mice. I computationally quantified expression within tumours and presented the data as a quickscore (QS) (Figure 5.2.5). QS is used for IHC semi-quantitation and is calculated as QS = staining intensity x proportion of positively-stained tissue. While transcription of Decorin and SPARC is upregulated in tumours, I find that protein expression is unchanged when *VANGL2* is lost. However, the expression of type I collagen is significantly increased in $VANGL2^{Flox/Flox}$ tumours over $VANGL2^{WT/WT}$. This represents another contradiction from chapter 4's expression analysis in in $VANGL2^{Flox/Flox}$ epithelial tissue, where I found type I collagen is significantly decreased in the healthy colonic epithelium. Additionally, laminin is significantly increased in $VANGL2^{Flox/Flox}$ tumours. I also found

that CTGF expression is increased when *VANGL2* is lost, which is in line with the mRNA expression data.

Furthermore, I also analysed the expression of downstream Wnt/PCP target c-Jun and its biologically active form phospho-c-Jun (Serine 73). It has previously been shown that inactivation of c-Jun reduces tumour number and size in the APC^{min} mouse model (Nateri, Spencer-Dene, & Behrens, 2005). I found no significant change in c-Jun or phospho-c-Jun expression in $APC^{flox/WT}$ $VANGL2^{Flox/Flox}$ tumours.

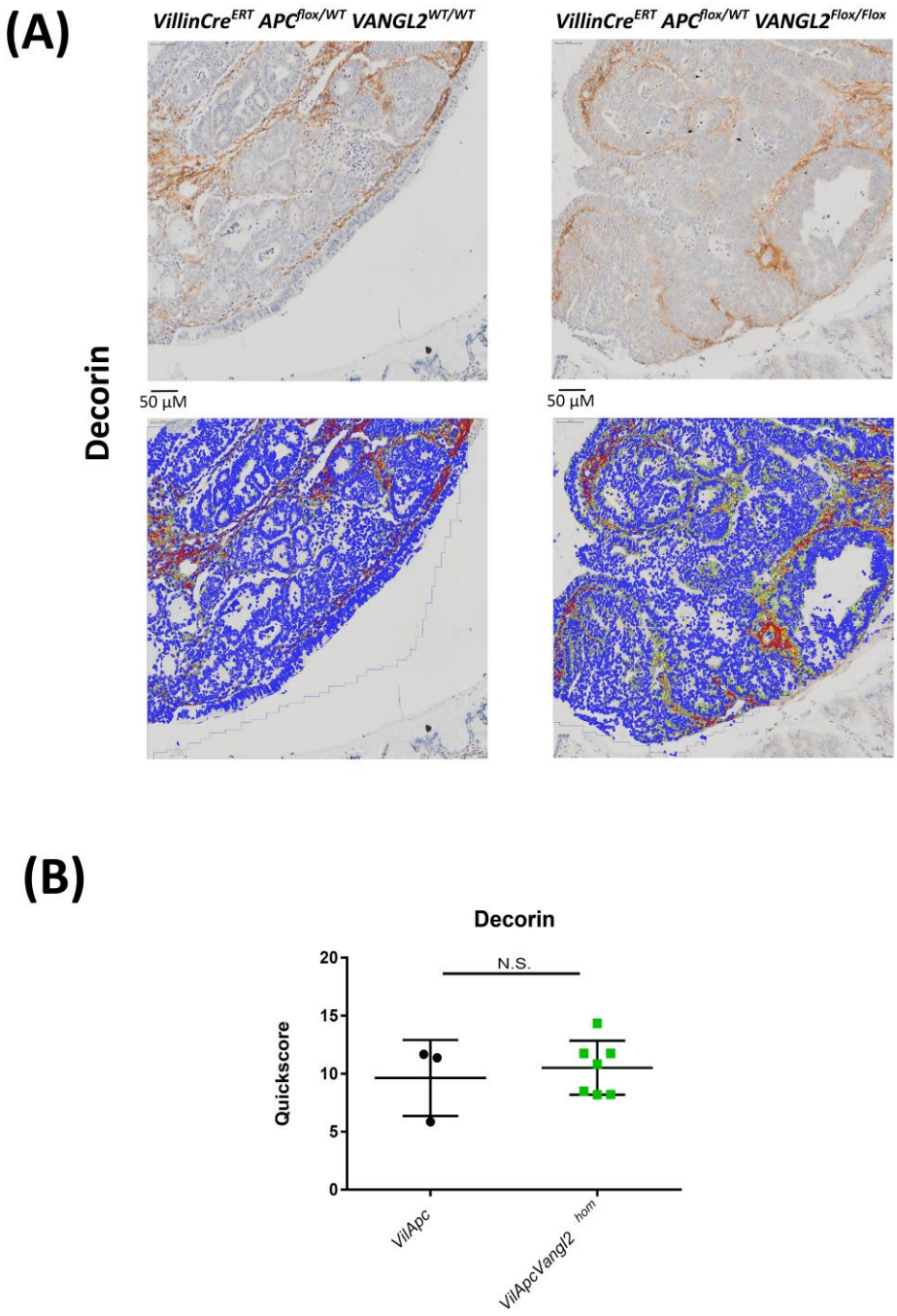


Figure 5.2.5 Immunohistological analysis of ECM-regulation by VANGL2 within the colonic tumour environment

(A) Fixed mouse colon from *VillinCre*^{ERT} *APC*$^{flox/WT}$ *VANGL2*$^{WT/WT}$and V*illinCre*^{ERT} *APC*$^{flox/WT}$ *VANGL2*$^{flox/flox}$ mice was stained for Decorin. Staining was visualised using DAB. Representative images used. Expression identification and categorisation (blue = nuclei, yellow = low expression intesity, orange = moderate expression, red = high expression). **(B)** Expression within colonic tumours was quantified computationally and presented as a quickscore. Quickscore = staining intensity x % area stained. Mean (SD); Student's t-test. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. At least 3 mice were used per genotype.

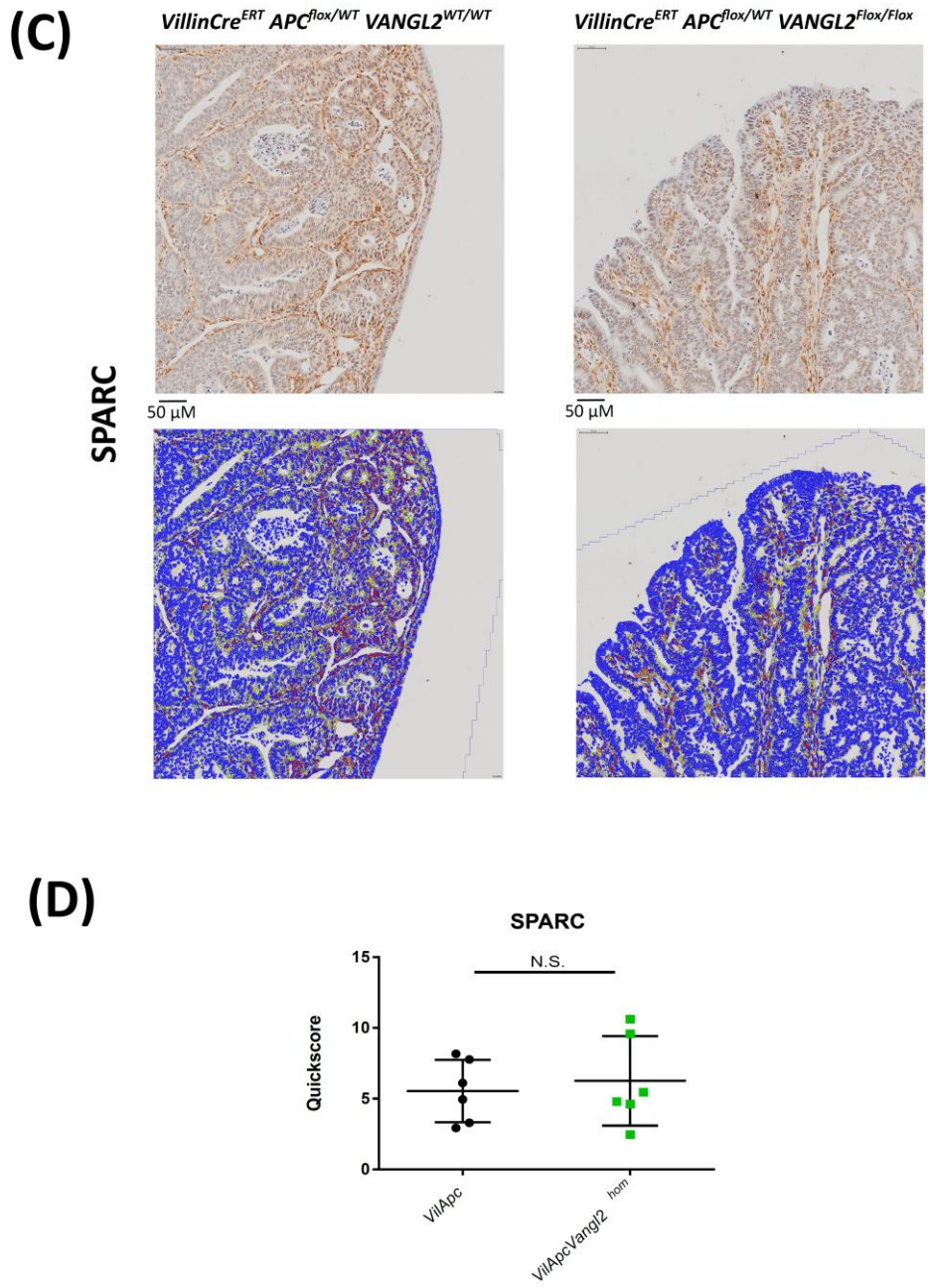


Figure 5.2.5 Immunohistological analysis of ECM-regulation by VANGL2 within the colonic tumour environment - *continued*

(C) Fixed mouse colon from *VillinCre*^{ERT} *APC*$^{flox/WT}$ *VANGL2*$^{WT/WT}$and V*illinCre*^{ERT} *APC*$^{flox/WT}$ *VANGL2*$^{flox/flox}$ mice was stained for SPARC. Staining was visualised using DAB. Representative images used. Expression identification and categorisation (blue = nuclei, yellow = low expression intesity, orange = moderate expression, red = high expression). **(D)** Expression within colonic tumours was quantified computationally and presented as a quickscore. Quickscore = staining intensity x % area stained. Mean (SD); Student's t-test. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. At least 3 mice were used per genotype.

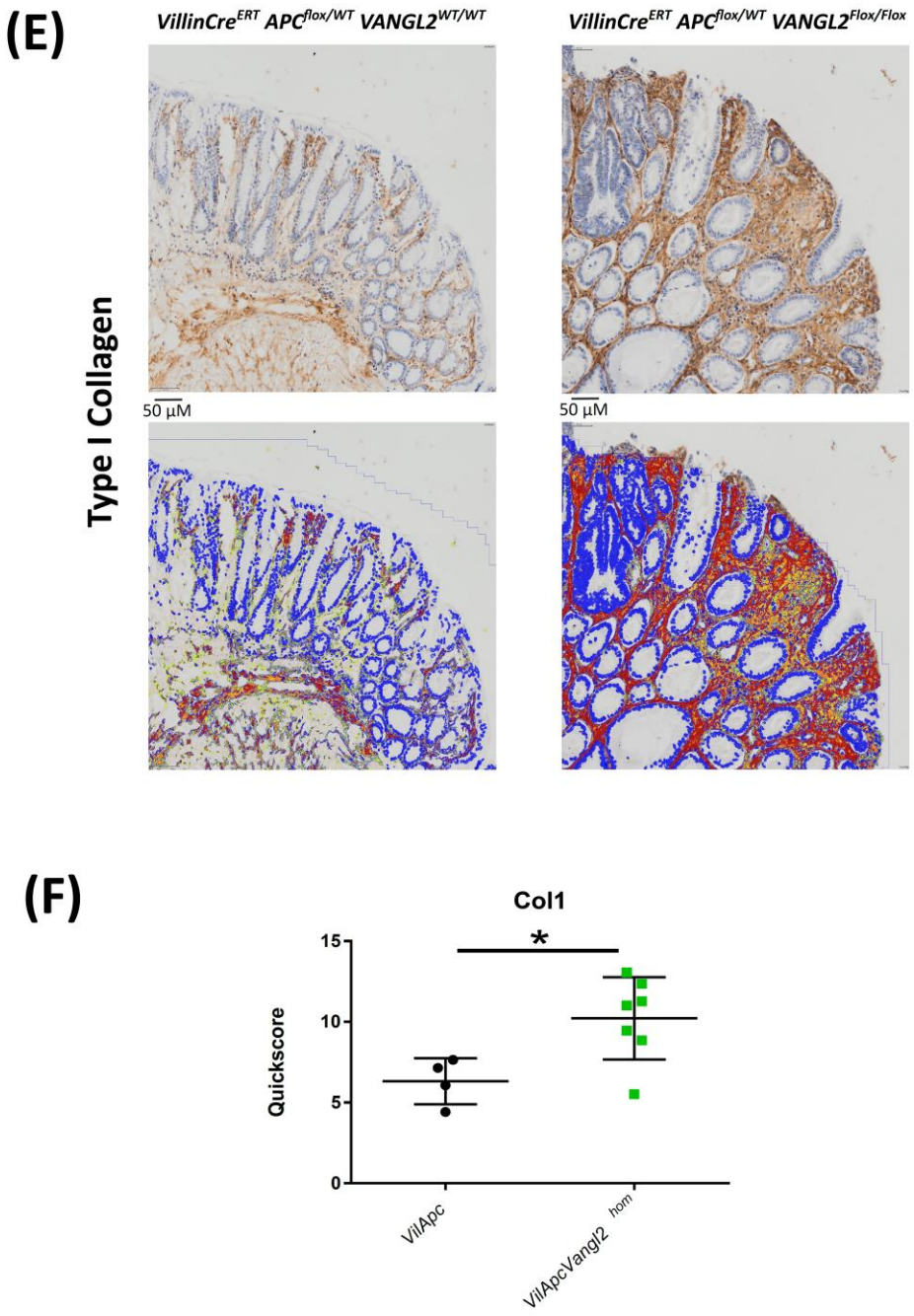
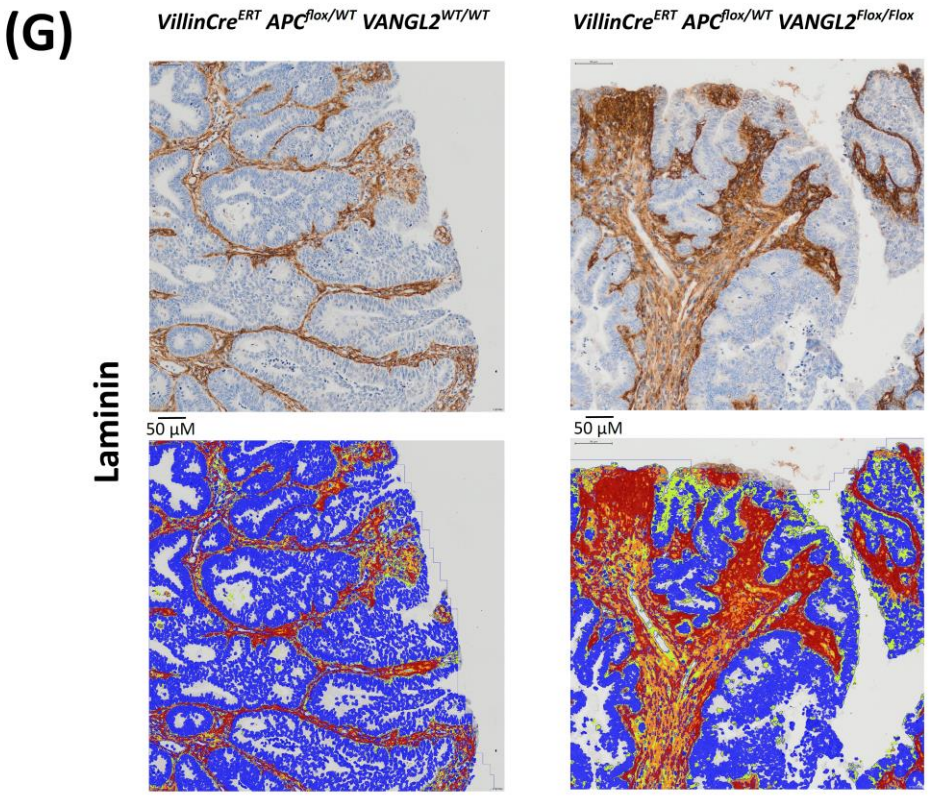


Figure 5.2.5 Immunohistological analysis of ECM-regulation by VANGL2 within the colonic tumour environment - *continued*

(E) Fixed mouse colon from *VillinCre^{ERT} APC$^{flox/WT}$ VANGL2$^{WT/WT}$* and *VillinCre^{ERT} APC$^{flox/WT}$ VANGL2$^{flox/flox}$* mice was stained for type I Collagen (Col1). Staining was visualised using DAB. Representative images used. Expression identification and categorisation (blue = nuclei, yellow = low expression intesity, orange = moderate expression, red = high expression). **(F)** Expression within colonic tumours was quantified computationally and presented as a quickscore. Quickscore = staining intensity x % area stained. Mean (SD); Student's t-test. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. At least 3 mice were used per genotype.



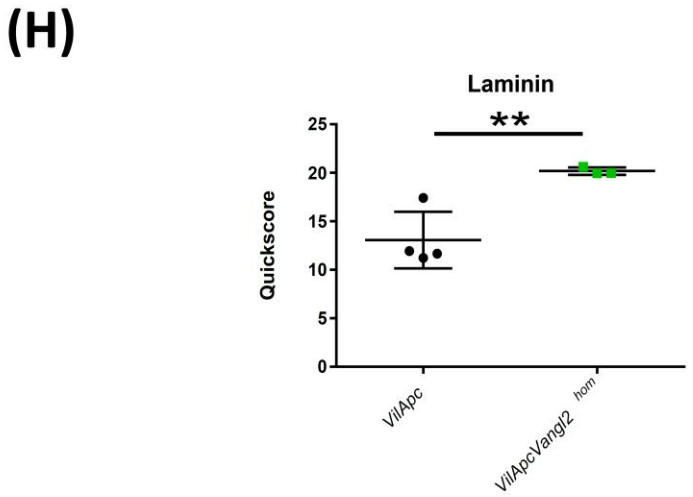


Figure 5.2.5 Immunohistological analysis of ECM-regulation by VANGL2 within the colonic tumour environment - *continued*

(G) Fixed mouse colon from *VillinCre*^{ERT} *APC*$^{flox/WT}$ *VANGL2*$^{WT/WT}$and V*illinCre*^{ERT} *APC*$^{flox/WT}$ *VANGL2*$^{flox/flox}$ mice was stained for Laminin. Staining was visualised using DAB. Representative images used. Expression identification and categorisation (blue = nuclei, yellow = low expression intesity, orange = moderate expression, red = high expression). **(H)** Expression within colonic tumours was quantified computationally and presented as a quickscore. Quickscore = staining intensity x % area stained. Mean (SD); Student's t-test. Mean (SD); Student's t-test. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. At least 3 mice were used per genotype.

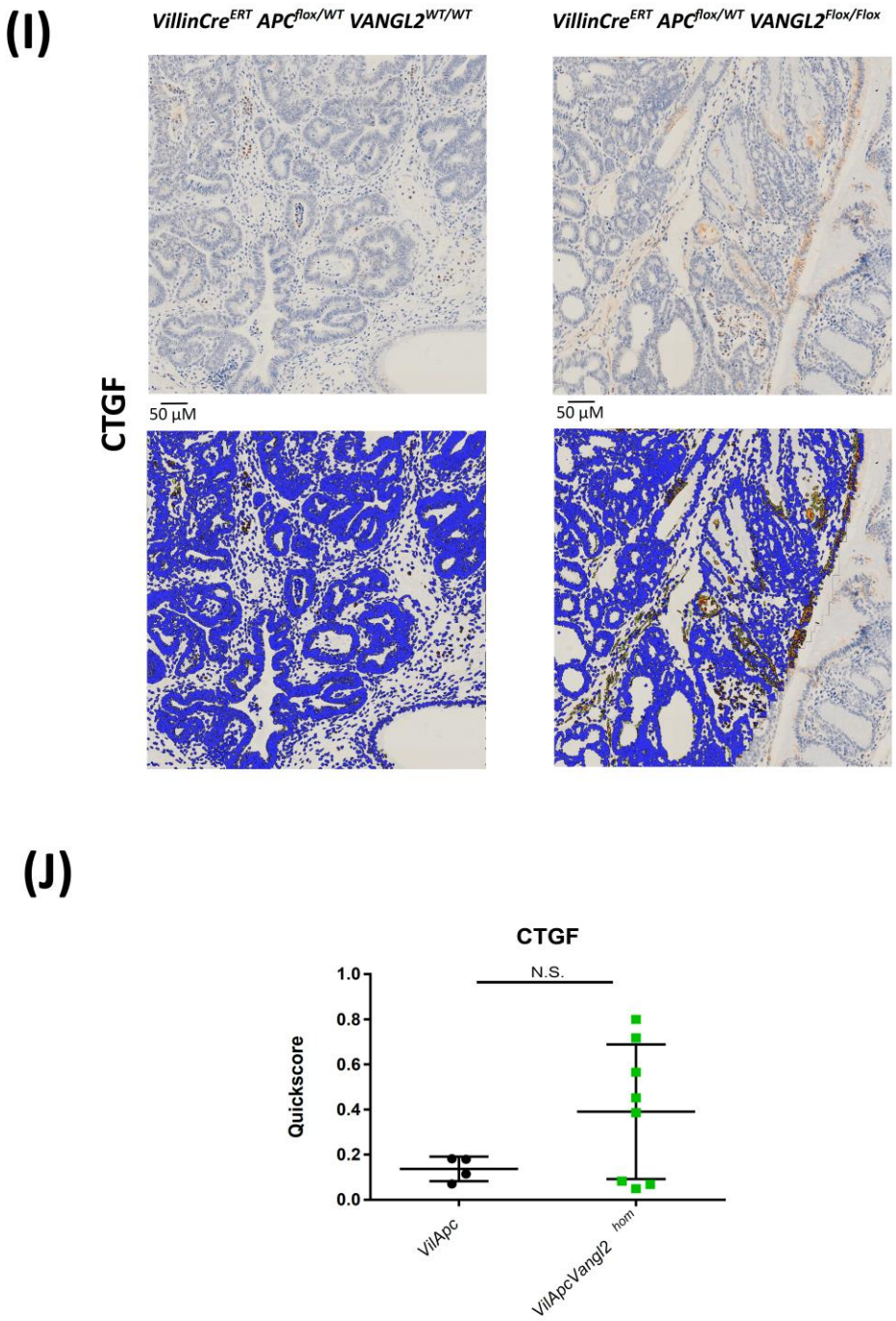


Figure 5.2.5 Immunohistological analysis of ECM-regulation by VANGL2 within the colonic tumour environment - *continued*

(I) Fixed mouse colon from *VillinCre*^{ERT} *APC*$^{flox/WT}$ *VANGL2*$^{WT/WT}$and V*illinCre*^{ERT} *APC*$^{flox/WT}$ *VANGL2*$^{flox/flox}$ mice was stained for CTGF. Staining was visualised using DAB. Representative images used. Expression identification and categorisation (blue = nuclei, yellow = low expression intesity, orange = moderate expression, red = high expression). **(J)** Expression within colonic tumours was quantified computationally and presented as a quickscore. Quickscore = staining intensity x % area stained. Mean (SD); Student's t-test. Mean (SD); Student's t-test. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. At least 3 mice were used per genotype.

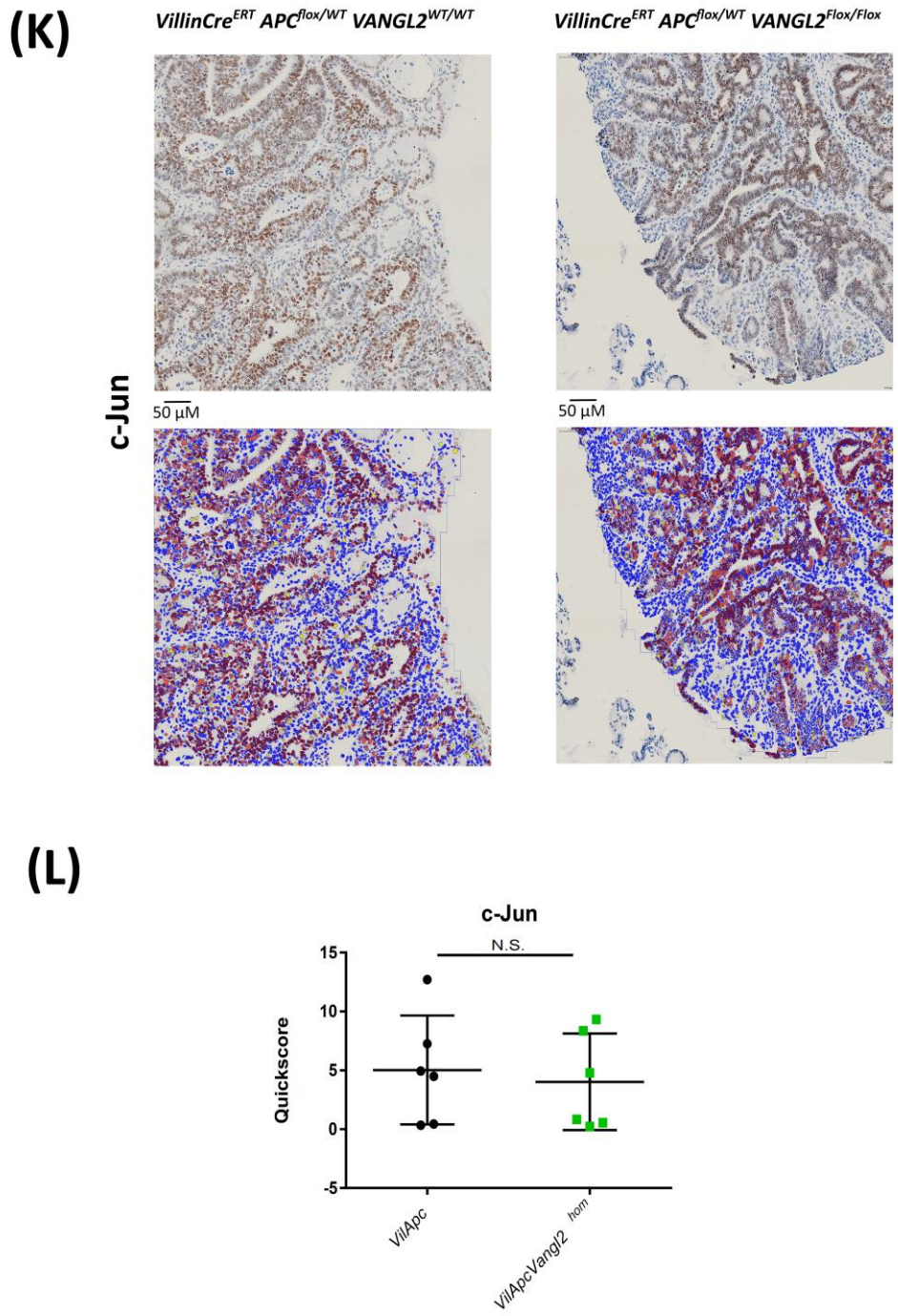
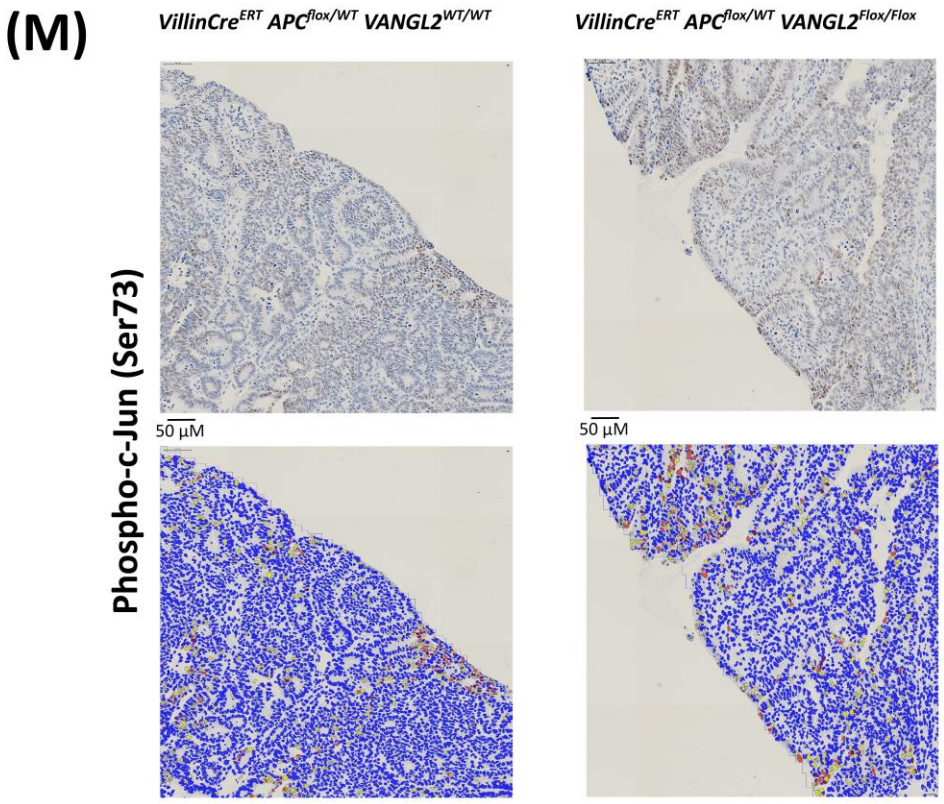


Figure 5.2.5 Immunohistological analysis of ECM-regulation by VANGL2 within the colonic tumour environment - *continued*

(K) Fixed mouse colon from *VillinCre^{ERT} APC$^{flox/WT}$ VANGL2$^{WT/WT}$* and *VillinCre^{ERT} APC$^{flox/WT}$ VANGL2$^{flox/flox}$* mice was stained for c-Jun. Staining was visualised using DAB. Representative images used. Expression identification and categorisation (blue = nuclei, yellow = low expression intesity, orange = moderate expression, red = high expression). **(L)** Expression within colonic tumours was quantified computationally and presented as a quickscore. Quickscore = staining intensity x % area stained. Mean (SD); Student's t-test. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. At least 3 mice were used per genotype.



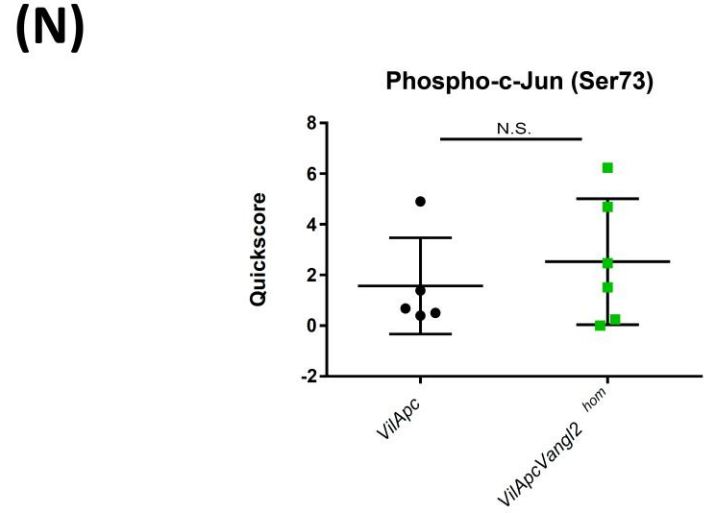


Figure 5.2.5 Immunohistological analysis of ECM-regulation by VANGL2 within the colonic tumour environment - *continued*

(M) Fixed mouse colon from *VillinCre^{ERT} APC$^{flox/WT}$ VANGL2$^{WT/WT}$* and *VillinCre^{ERT} APC$^{flox/WT}$ VANGL2$^{flox/flox}$* mice was stained for Phospho-c-Jun (Ser73). Staining was visualised using DAB. Representative images used. Expression identification and categorisation (blue = nuclei, yellow = low expression intesity, orange = moderate expression, red = high expression). **(N)** Expression within colonic tumours was quantified computationally and presented as a quickscore. Quickscore = staining intensity x % area stained. Mean (SD); Student's t-test. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. At least 3 mice were used per genotype.

5.2.6 Intestinal organoids as an *ex vivo* tool for investigating *VANGL2*-mediated signalling

While the *in vivo* studies so far present an ideal representation of the epithelial phenotype in functional loss of *VANGL2*, it comes with common issues such as the time taken to generate adult mice and intestinal tumours, as well as issues with the presence of cell types in the stroma and inflammatory processes which can confound data in evaluating epithelial phenotypes. Traditionally human CRC cell lines (such as HCT 116 or DLD1 cells) have been used as *in vitro* models. However, these cells do not typically reflect the cell-type of the tissue from which they were derived. They have often undergone a vast number of passages resulting in adaptations to culture. There are also limitations in the amount of pathological data for each of the cell lines available. Therefore, I decided to develop a more physiological epithelial culture system.

Epithelial organoid culture allows recapitulation of tissues into models of epithelial homeostasis or disease. It was found that epithelial stem cells (within intestinal crypts) can undergo long-term culture when dissociated from tissue and placed in Matrigel (a laminin-rich basement membrane replacement (Sato et al., 2009). It was later found that adenoma/adenocarcinoma tissue could also be

cultured in this way (Sato, Stange, et al., 2011). I decided, therefore, to utilise adenoma organoid culturing to investigate pathways altered by loss of *VANGL2* in colonic adenomas.

Intestinal organoid cultures were first prepared from healthy epithelial crypts from WT mice (Figure 5.2.6A). Dissected colonic tissue was agitated for 30 minutes in 25 mM EDTA at 4C. Extracted crypts were plated in Matrigel with growth factors necessary for colonic organoid growth (EGF, Noggin, R-Spondin1, and Wnt3a). Self-organising intestinal crypt structures are formed within a week. Intestinal adenoma organoid culture was performed by extraction of tumours from *VillinCre^{ERT} APC$^{flox/WT}$* mice three months after induction with tamoxifen (Figure 5.2.6B). Tumours were isolated and digested into single cells using TrypLE Express (Gibco). Single cells are plated in Matrigel. Organoids were cultured without R-spondin1 growth factor or Wnt ligand, required in colonic crypt organoid culture, to select for cancer cells (which have constitutive β-catenin stabilisation due to loss of APC, which typically acts to turn over b-catenin). After a week, organoids form into large hyperproliferative cysts.

Adenomas from *VillinCre^{ERT} APC$^{flox/WT}$ VANGL2$^{WT/WT}$* and *VillinCre^{ERT} APC$^{flox/WT}$ VANGL2$^{flox/flox}$* mice were collected and cultured as organoids in Matrigel (Figure

5.2.6C). There was no observed difference in viability or architecture in the *VANGL2*-deficient organoids. This could be explained by the loss of APC, which results in the elevation of canonical Wnt signalling. Established organoids were collected and consequently used in qRT-PCR analysis of gene expression. As expected, *VANGL2*$^{flox/flox}$ organoids had ablated *VANGL2* expression ($p = 0.040$) (Figure 5.2.6D). Expression of canonical Wnt signalling targets was analysed (Figure 5.2.6E). No significant change in mRNA expression was found in the *VANGL2*$^{flox/flox}$ organoids. This is as anticipated, due to the Wnt/B-catenin signalling hyperactivation from APC loss. Next, I assayed targets of non-canonical Wnt signalling: SPARC, Decorin, Fos1, and PKCθ (Figure 5.2.6F). No significant changes in expression were found in these targets, suggesting that *VANGL2* may not be regulating the non-canonical pathways in murine adenomas. Finally, the expression common targets of Hippo (YAP/TAZ) signalling, which require non-canonical Wnt activation, were quantified: comprised of WNT5B, BMP4, CYR61, IGFPB4, and CTGF (Figure 5.2.6G). I found that BMP4 expression was significantly downregulated in the *VANGL2*$^{flox/flox}$ organoids ($p = 0.033$). As with the *in vitro* tumour analyses, CTGF was found to be upregulated when *VANGL2* expression is lost.

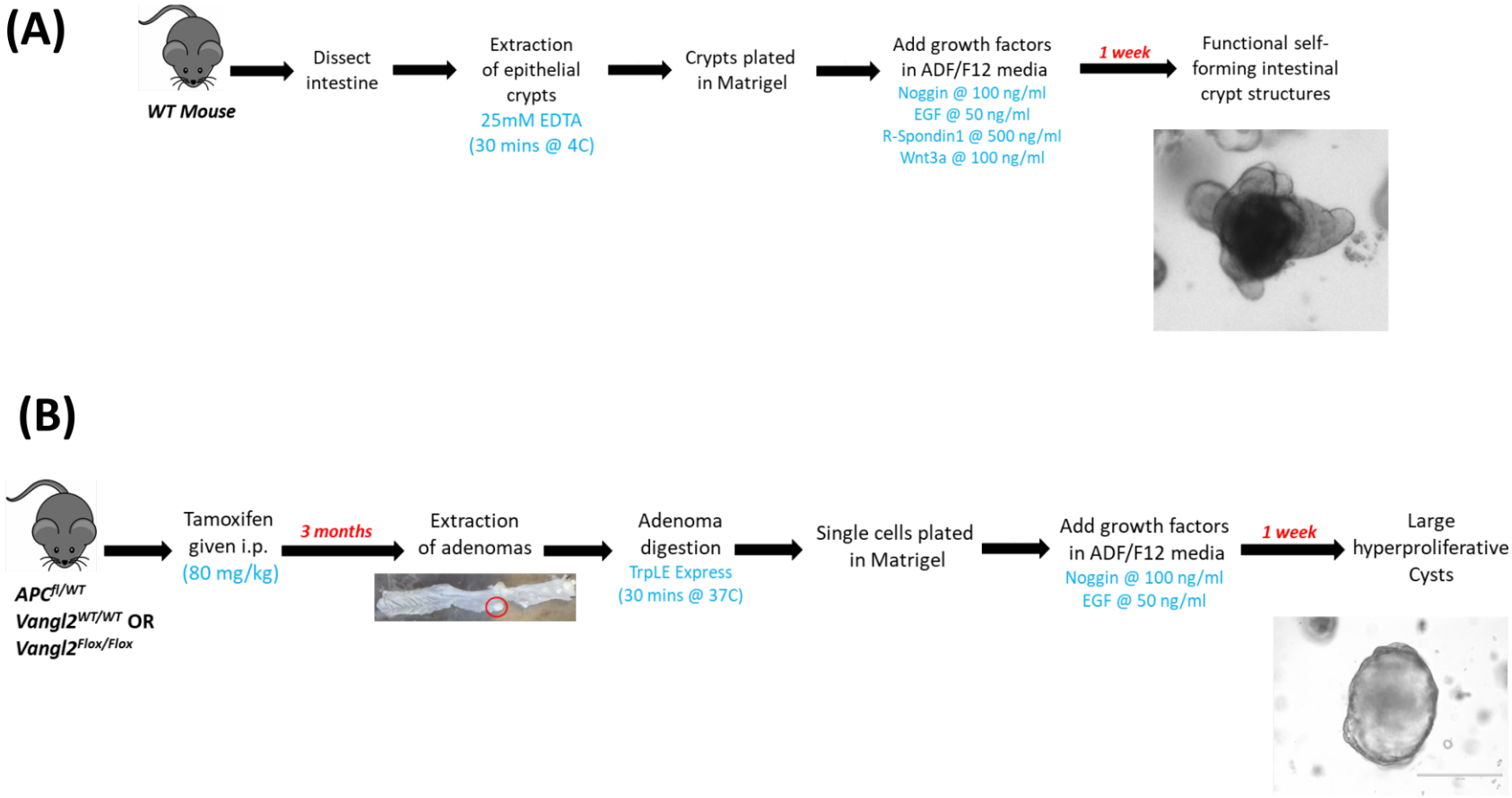


Figure 5.2.6 Intestinal organoids as an *ex vivo* tool for investigating VANGL2-mediated signalling

(A) Colonic crypt organoid culture strategy. Dissected intestine from WT mice are incubated in 25mM EDTA for 30 minutes with agitation at 4C. Following this, crypts are extracted with forcefull pipetting and crypts plated in Matrigel. Growth factors necessary for colonic organoid culture are provided in the media: Noggin (100 ng/ml, EGF 50 ng/ml, R-Spondin1 500ng/ml, Wnt3a 100 ng/ml. Crypts form organoid structures after 1 week, recapitulating crypt morphology seen *in vivo*. **(B)** Colonic adenoma organoid culture strategy. VillinCreERT APCFlox/WT Vangl2WT/WT and Vangl2Flox/Flox mice are induced with Tamoxifen and aged for 3 months to allow for adenoma growth. Dissected adenomas are digested with TrypLE Express (Gibco) for 30 mins at 37C and single cells plated in matrigel. Adenoma organoids are grown without Wnt signalling stimulation from R-Spondin1 or Wnt3a to select for Wnt hyperactive APC-null organoids. After 1 week large hyperproliferative crypts are formed from adenoma cells.

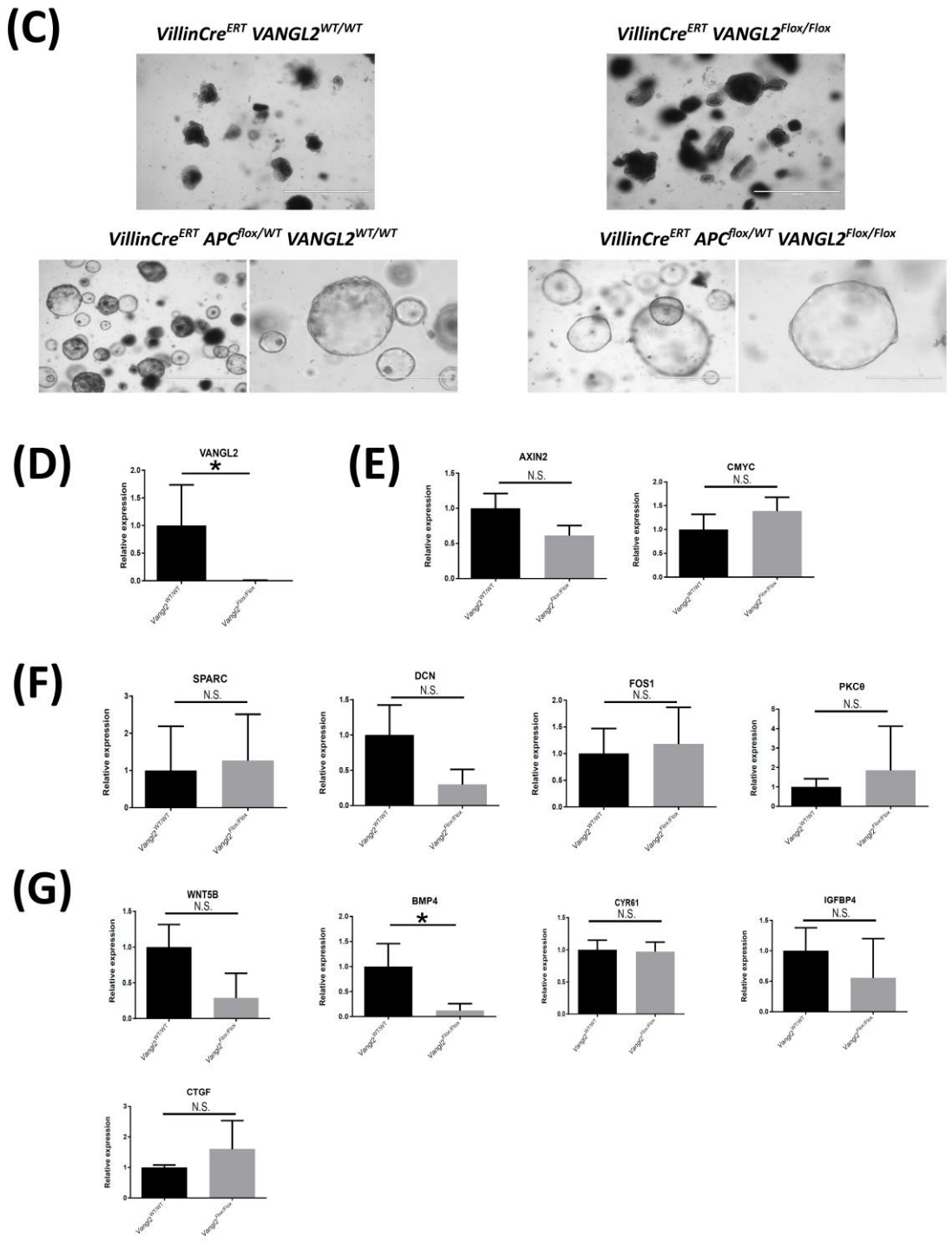


Figure 5.2.6 Intestinal organoids as an *ex vivo* tool for investigating VANGL2-mediated signalling - *continued*

(C) Representative images of colonic adenoma organoids taken from *VillinCre^{ERT} APC$^{flox/WT}$ VANGL2$^{WT/WT}$* and *VillinCre^{ERT} APC$^{flox/WT}$ VANGL2$^{flox/flox}$* mice, as well as organoids taken from the equivalent non-mutant APC mice (featuring a non-cystic phenotype). Organoids created by culturing intestinal adenoma tissue in matrigel. Cancer cells form 3D 'spheroids'. **(D-G)** Organoids were removed from matrigel and RNA extracted. cDNA was synthesised and qRT-PCR performed for **(D)** VANGL2, **(E)** canonical Wnt signalling targets, **(F)** non-canonical Wnt signalling targets, and **(G)** YAP/TAZ signalling targets. For each gene, expression values are relative to the *WT*. All expression normalised to Ppia. Student's t-test; Mean (SD) values shown. Mean (SD); Student's t-test. N.S. (not significant) = $p > 0.05$ * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Colonic tumour organoids derived from 3 seperate mice were used per genotype.

5.3 Discussion

As previously discussed, *Piazzi* et al found that *VANGL2* overexpression in CRC cells decreased proliferation and colony formation (Piazzi et al., 2013). This data would suggest, therefore, that *VANGL2* plays a role in restricting tumour initiation in CRC. In breast cancer, *VANGL2* was shown to be overexpressed in tumour over non-tumour samples. It was also shown that in breast cancer cell xenografts that *VANGL2*-overexpressing cells causes a dramatic reduction in time for tumour development, suggesting that *VANGL2* plays a role in promoting tumour initiation. It was found that this effect could be ameliorated by disrupting JNK signalling (Puvirajesinghe et al., 2016). Given that from TCGA data we can see *VANGL2* expression is associated with DFS in CRC patients, it may be that JNK signalling also plays a part CRC progression. This is significant as it suggests that *VANGL2* can function as a prognostic marker in breast cancer, while *VANGL2*-mediated JNK signalling could be a therapeutic target in the future.

In chronic lymphoid leukaemia (CLL), *VANGL2* is overexpressed, and it is shown that PCP pathway components are required for migration and cellular invasiveness (Kaucká et al., 2013). As well as this, high expression of PCP genes FZD3, FZD7, and PRICKLE1 (but not *VANGL2*) is associated with worsened CLL survival. In

2010, it was shown that loss of *VANGL2* in cancer cells promotes ECM invasion, and this increased invasion was a result of directed migration, and that this cancer progression was MMP-dependent (Cantrell & Jessen, 2010). In other research it was shown that *VANGL2* regulates degradation of the ECM in human fibrosarcoma cells (B. Blairanne Williams et al., 2012).

Overall, the role of *VANGL2* (and PCP signalling) in tumour development appears highly context dependent. Within the limited *in vitro* analyses on *VANGL2* in CRC published so far, it seems that *VANGL2* plays a role in tumour initiation. This shows, therefore, that *in vivo* analyses of *VANGL2* are needed for elucidating its role within the colorectal cancer context. My observations suggest that *VANGL2* mediates colonic tumour initiation.

I created a *VANGL2* loss-of-function (LOF) intestinal cancer model, and found that mice with partial or full *VANGL2* LOF did not have altered survival when compared to the *WT* animals. This is the first time that an inducible knockout of *VANGL2* has been used to look at cancer development *in vivo*. Previous work looking at survival in breast cancer found *VANGL2* is associated with poorer prognosis (Puvirajesinghe et al., 2016).

The fact that *VANGL2* LOF mice develop significantly less colonic tumours compared to WT mice suggests that *VANGL2* mediates tumour initiation. This is in contrast from the work by Piazzi et al*,* where they found *VANGL2* expression inversely correlated with CRC cell proliferation and colony formation *in vitro* (Piazzi et al., 2013). However, in using an *in vivo* model of breast cancer, it has been found that *VANGL2* acts as an activator of tumour development, in agreement with my data (Puvirajesinghe et al., 2016). Cancer models can recapitulate some of the aspects seen during tumour growth including metastasis, invasion, and angiogenesis, which are typically lost when working with cell lines. Given that loss of *VANGL2* is associated with tumour promotion in both my cancer model and in the previously mentioned research in breast cancer, we can be confident that *VANGL2* is worthy of investigation as a mediator of tumour development in other cancer models. However, the lack of tumour progression to invasion or metastasis does not preclude *VANGL2* from regulating these processes. Using more models of CRC which recreate more advanced tumours, I could analyse how *VANGL2* impacts the invasiveness and/or metastatic potential of intestinal tumours (Marsh et al., 2008).

The restriction of colonic tumour number was not observed in the heterozygous LOF *VANGL2* mice. This is similar to data showing *VANGL2* acts genetically dominant in development (E. Torban et al., 2008). In development, both copies of VANGL2 must be inactivated for a phenotype to occur, and my data suggests

that VANGL2 displays a similar dominance in the context of colonic adenoma development.

Interestingly, the *VANGL2* LOF tumour initiation phenotype found in the colon was not seen in the small intestine. Colon-specific tumour regulation has been found in APC^{min} mice before; it is likely that, in mice, a different set of genetic alterations are needed for small intestinal tumour development compared to the colon (Lefebvre et al., 1998; Wheeler, 2002). The lack of a small-intestinal phenotype in this model could be as a result of the absence of prominent VANGL2 and PTK7 co-localisation in the small intestine. This data may, therefore, signal that *VANGL2* is a colon-specific promoter of tumour development. Interestingly, a Wnt/PCP factor downstream of *VANGL2* had a contrasting effect. In another intestinal cancer mouse model, RhoA was inactivated within the intestinal epithelium, leading to an increase in small intestinal tumours but no effect on colonic tumours (Rodrigues et al., 2014). As the phenotype observed here is specific to colonic tumour initiation, we could use a colonic-specific tumour model by inducing colitis-associated dysplasia in APC mutant mice by treating with dextran-sodium sulphate (DSS), or by treatment with the carcinogen azoxymethane (AOM) (Cooper et al., 2001; Schwitalla et al., 2013).

It may be that *VANGL2* is only a promoter of tumour development in the colon. It was also found that colonic tumour burden was significantly decreased in the $APC^{flox/WT}$ $VANGL2^{Flox/Flox}$ mice. However, as average tumour size was found to be unchanged, this effect is a result of increased tumour number. This implies that *VANGL2* deficiency modifies adenoma initiation, but is not a modifier of adenoma growth. In contrast, the aforementioned paper examining *VANGL2* in breast cancer found that suppressed expression of *VANGL2* abrogated tumour growth (Puvirajesinghe et al., 2016). These data, therefore, supports the theory that *VANGL2* functions as an oncogene in colonic tumour initiation.

As previously mentioned, the ECM component Decorin was found to have increased expression in *VANGL2* LOF colonic tumours. As Decorin has previously been shown to act as a suppressor of intestinal tumour initiation *in vivo,* upregulation of Decorin in the intestinal tumours following *VANGL2* loss may be the reason I have observed abrogated tumour initiation in my model (Bi et al., 2008). Fellow ECM protein SPARC was upregulated in *VANGL2* LOF tumours, but has previously been shown to be pro-tumourigenic in murine intestinal cancer model (Sansom, Mansergh, Evans, Wilkins, & Clarke, 2007). This is in contrast to what is expected, as we see that loss of *VANGL2* in my intestinal tumour model reduces tumourigenesis. I have found TIMP1 expression is increased in *VANGL2* LOF tumours. TIMP1 has been found to suppress angiogenesis in fibrosarcoma cells, suggesting that *VANGL2* deficient CRC

may be more resistant to angiogenesis (Ardi, Kupriyanova, Deryugina, & Quigley, 2007). This would support data I presented earlier showing that CRC patients with high *VANGL2* expression have faster disease recurrence (a phenotype which could be caused by angiogenesis). The increases in ECM components found here contrasts with the expression levels found in *VANGL2* LOF epithelium, where they are decreased. This suggests that these ECM components may act as tumour markers of improved DFS, if indeed these changes are as a result of *VANGL2*-deficiency. CTGF transcripts and protein expression are increased in tumours when *VANGL2* is lost, and this transcriptional change is replicated in tumour organoids. This is a strongly indicative finding, as it has previously reported effects in inhibiting CRC metastasis and high expression correlating with higher disease-free survival (Bi et al., 2008). This correlates with my finding that patients with high *VANGL2* expression experience higher disease-free survival. It may be that the epithelial 'niche' in tumours with high *VANGL2* expression may be primed for invasiveness/metastasis through reduction in CTGF levels. In addition, I observe increased deposition of type I collagen, the most abundant interstitial protein of the ECM, in *VANGL2* LOF tumours. Accumulation of type I collagen is associated with a fibrotic phenotype, as Kauppila et al found in breast cancer that malignant breast cancer tumours display increased expression of type I procollagen mRNA in the fibroblastic cells of the stroma (Pathol, Stenback, Risteli, Jukkola, & Risteli, 1998).

While my analyses looking at c-Jun and phospho-c-Jun (Serine 73) found no significant change was found when *VANGL2* is lost, suggesting *VANGL2* does not regulate downstream JNK activity in tumour development, Wnt/PCP signalling can function through RhoA/ROCK signalling and has been implicated in the invasiveness/migratory ability of CRC cells (Bi et al., 2008).

I have also established intestinal organoid culture as a tool for investigating *VANGL2*-mediated signalling. This allows me to utilise the *VANGL2* LOF tissue to assess tumour-regulating signalling pathways without the lengthy process of obtaining animal tissue. This *ex vivo* technique will allow close to physiological CRC conditions. However, limitations apply to this method as organoid cultures only represent the epithelial cell population of an intestine, while the basement membrane matrix is provided by Matrigel (Corning). It has been shown that matrix-organoid interactions can alter cellular behaviour, so advancements in technology to allow as close to physiological conditions as possible in the matrix would be welcome (Pastuła et al., 2016). From assays looking at Hippo (YAP/TAZ) target gene transcription, I found that BMP4 and WNT5B were downregulated in *VANGL2* LOF tumour organoids. BMP4 has been shown to promote terminal differentiation and apoptosis in CRC stem cells, while also increasing chemosensitivity (Lombardo et al., 2011). Downregulation of tumour BMP4 expression, therefore, does not immediately explain why we see reduced colonic tumour initiation in the *VANGL2*

LOF intestinal cancer model. However, the evidence for the effect of BMP4 in CRC development is conflicting. BMP4 expression is seen to be highly enriched at the invasive fronts of human CRCs, suggesting a role played by BMP4 in invasion (Voorneveld et al., 2015). As well as this, BMP4 is reported to support lung cancer tumourigenesis and metastasis in murein allograft models (J. S. Kim, Kurie, & Ahn, 2015). I also see downregulation of WNT5B, implicated in CRC migration and invasion through JNK signalling, in *VANGL2* LOF tumours (Y. Zhang et al., 2016). This would suggest that *VANGL2* could be again responsible for maintaining an epithelial niche primed for invasiveness, giving a possible explanation why CRC patients with high *VANGL2* expression are associated with reduced DFS. In the future, this organoid culture system will allow further analysis of *VANGL2*-mediated signalling and binding partners in colonic tumourigenesis. It would also be interesting to investigate gene targets upstream of the genes assessed here, such as those involved in Wnt/PCP signalling.

Overall, these data indicate that *VANGL2* is an important factor in colonic tumour development. I found that *VANGL2* in the mouse colon promotes tumour initiation in the $APC^{flox/WT}$ model. ECM components key to CRC development were found to be upregulated when *VANGL2* is lost, including type I Collagen, Laminin, and CTGF. This suggests that *VANGL2* is involved in regulating levels of these proteins in tumours. Alternatively, loss of *VANGL2* may result in more indirect changes to ECM

component stability. *VANGL2* LOF tumour organoids also showed upregulation of CTGF, and interestingly, downregulation of BMP4. *VANGL2* is involved in promoting colon tumour development and this is may be via regulation of the ECM and modulation of CTGF and BMP4. This theory could be tested by analysing BMP4 secretion and activation within WT and *VANGL2*-deficient tumours.

Chapter 6: Discussion

VANGL2 is a critical mediator of development through its role in morphogenic Wnt/PCP signalling. This key regulator is important in a number of different functions, from its role in proper neural tube closure, to eye development (Y.-P. Lei et al., 2010; Leung et al., 2016). Often, key developmental signals are utilised in adult tissues to maintain homeostasis. However, when these key signals are aberrantly activated/repressed they can lead to the development of disease. One of the best characterised morphogenesis pathways is canonical Wnt signalling, where it is required for maintenance of many adult tissues, and perturbations in this signalling cause many human diseases including cancer (Nusse, 2005). While the canonical Wnt signalling pathway is a key activator of colorectal cancer (CRC), there is limited research around the non-canonical pathways (such as Wnt/PCP signalling) within this context. While maintenance of proper polarity has been implicated in protecting against CRC progression (as Langlois and colleagues found with PTEN phosphatase), no such evidence exists for the planar cell polarity pathway (Langlois et al., 2010).

Current literature provides little insight as to how *VANGL2* functions in the context of the adult intestine, with the majority of recent studies examining the role of *VANGL2* in mammalian tissue morphogenesis during embryonic development

(Findlay et al., 2016; L. Li et al., 2017; Obara, Suzuki, Irie, & Shibata, 2017; Yang et al., 2017). There are, however, some reports indicating that *VANGL2* plays important roles in both lung and kidney podocyte homeostasis, in which it is indicated that *VANGL2* is necessary for proper tissue growth in the adult and could be critical for tissue repair in human adults (Poobalasingam et al., 2017; Rocque et al., 2015).

In this thesis, I have defined the role *VANGL2* plays in intestinal homeostatic mechanisms and in cancer using human CRC patient transcriptomic databases, patient tumour samples, murine intestinal *VANGL2* loss-of-function, intestinal cancer models, and colonic adenoma organoids. This body of work consists of 2 major themes; the first investigates the role of *VANGL2* in the healthy intestinal epithelium and pathways controlled by it, and the second examines *VANGL2* as a mediator of colorectal cancer.

6.1 *VANGL2* as a regulator of intestinal epithelial homeostasis

VANGL2 plays a critical role in mammalian development through establishment of planar cell polarity (PCP). It is essential for proper morphogenesis of various epithelial structures including the neuroepithelium, where loss-of-function (LOF) mutations in *VANGL2* have been identified in humans with neural tube defects (NTDs) (Kibar, Vogan, et al., 2001; Y.-P. Lei et al., 2010; van Abeelen & Raven, 1968). *VANGL2* is required for proper fore-stomach morphogenesis and it is also expressed in the intestinal tube during development (Cervantes et al., 2009; Satoh et al., 2008; Elena Torban et al., 2007). Pathways essential during embryonic development of tissue are often key regulators of that tissue in the adult, be it regulating cellular behaviour or regenerative activation in response to tissue insult. However, there has been limited research conducted on *VANGL2* in mediating healthy adult tissue. Poobalasingam et al found that heterozygous loss-of-function of *VANGL2* results in impaired lung function, as well as markers of lung epithelial tissue damage. An interaction between *VANGL2* and lung function was also found in smokers. The implication of this study is that *VANGL2* is required for lung homeostasis (Poobalasingam et al., 2017). Looking at the adult corneal epithelium, Findlay et al showed that knockdown of

VANGL2 disrupted cellular migration in cells and *in vivo* through loss of planar alignment (Findlay et al., 2016). These studies again suggest that *VANGL2* plays a role in supporting tissue homeostasis in the adult.

In examining *VANGL2* in the adult mouse colonic epithelium for the first time, I found that *VANGL2* is expressed along with Wnt/PCP ligand WNT5A. In the developing limb bud, it has been shown that WNT5A can induce *VANGL2* phosphorylation of serine/threonine residues, and that this phosphorylation can regulate *VANGL2*'s PCP activity (B. Gao et al., 2011). WNT5A has been reported to be essential for regeneration of colonic crypts by, paradoxically, inhibiting crypt SC proliferation. This is caused by WNT5A activation of transforming growth factor-β (TGF- β) signalling, thereby establishing the importance of WNT5A in intestinal homeostasis (Miyoshi, Ajima, Luo, Yamaguchi, & Stappenbeck, 2012). I also showed that *VANGL2* co-localises with PTK7 within the colon. *VANGL2* and PTK7 have been previously shown to genetically interact in vertebrate development as well as display similar abnormal PCP phenotypes when either is overexpressed (Hayes et al., 2013; Lu et al., 2004). Martinez et al showed that PTK7 and ROR2 physically interact to activate JNK and cellular migration, and that this is potentiated by WNT5A (Martinez et al., 2015). The presence of this Wnt/PCP 'signalling hub' within the murine colon supports the theory that *VANGL2* participates in homeostatic signalling within this tissue. Furthermore, I describe for the first time that *VANGL2* is also expressed within

the healthy human colon, and co-localises with PTK7. PTK7 has been shown to promote Wnt/PCP signalling in vertebrate development (Hayes et al., 2013). Finding similar component arrangement within murine and human epithelium suggests that Wnt/PCP signalling is comparable between species. While murine models are regularly used to investigate Wnt signalling, the highly tissue-dependant effects of Wnt/PCP signalling may limit comparisons across species.

When *VANGL2* is functionally lost from the murine colonic epithelium, I found no architectural disorganisation or alteration to proliferation. This suggests that *VANGL2* does not play an essential role in intestinal integrity or mucosal turnover in the adult. However, *VANGL2* may be required for homeostatic mechanisms not tested within this thesis. For example, *VANGL2* may modulate regeneration or injury response in the intestine. This is similar to what is observed in lung epithelial damage previously mentioned and in podocytes where deletion of *VANGL2* leads to an exacerbation of the damage response (Rocque et al., 2015). Inducing colitis using DSS in my *VANGL2* LOF mouse model and assessing regenerative capacity would allow interrogation of how *VANGL2* mediates response to mucosal damage in the colon (Chassaing, Aitken, Malleshappa, & Vijay-Kumar, 2014).

Wnt/PCP signalling is typically represented by two downstream pathways. The first, activation of RhoA which leads to ROCK activation, resulting in cytoskeletal rearrangements. The second, activation of JNK and subsequent activation of c-Jun, part of the AP-1 transcription factor complex. I demonstrate that ROCK1 and ROCK2 are upregulated in the murine colonic epithelium when *VANGL2* is lost, suggesting that *VANGL2* may act to restrict RhoA/ROCK signalling or that downstream components of the pathway are over expressed to compensate for *VANGL2* loss. I also found that AP-1 subunits FRA1 and FRA2 are downregulated when *VANGL2* is lost. Further to this, I showed that AP-1 subunits c-Jun and Fos1 are also downregulated when *VANGL2* is lost. This suggests that *VANGL2* acts to promote the AP-1 transcription factor complex through Wnt/PCP signalling, potentially acting as a point of positive autoregulation. There may also be a signalling equilibrium where *VANGL2* mediates Wnt/PCP/AP-1 signalling over Wnt/PCP/ROCK signalling. Supporting this theory, I found that AP-1 transcriptional targets Decorin and COL1A2 are downregulated when *VANGL2* is lost. However, further work is necessary to evaluate RhoA activity and its downstream effects on cytoskeletal rearrangement. Downstream Wnt/PCP signal activation in my *VANGL2* LOF model could be further measured by looking at activation of JNK/cJun and RhoA/ROCK signalling. Examination of JNK phosphorylation is one of the most well used readouts of Wnt/PCP signalling and has previously been used to show that JNK activation is reduced when *VANGL2* is knocked down in breast cancer cells, while cJun

phosphorylation, at Serine 63 and Serine 73, has also been used as a readout for PCP signalling (Puvirajesinghe et al., 2016; Shafer et al., 2011). Active-RhoA specific pulldowns have also been used to quantify RhoA activity in cells (for example in examining the PCP component RYK), however this has never been applied to look at *VANGL2* modulation of RhoA activity (Macheda et al., 2012). However, *VANGL2* has been found to control the localisation of RhoA and ROCK1 in the developing myocardium, while phosphorylation of downstream targets of RhoA/ROCK such as MYPT696 has been used as an indicator of activity. MYPT696 localisation was also found to be under the control of *VANGL2* in the developing myocardium (Phillips et al., 2008, 2005).

Another non-canonical Wnt signalling pathway is implicated in my *VANGL2* LOF model. Wnt/Ca^{2+} signalling (involved in cellular behaviours such as proliferation and migration) controls intracellular Ca^{2+} levels, which can activate the Protein Kinase C (PKC) family of proteins. I discovered that one these proteins, PKCθ, is downregulated when *VANGL2* is lost in the colonic epithelium, and other Ca^{2+} signalling transcripts are also altered. No knowledge exists on interactions between *VANGL2* and Wnt/Ca^{2+} signalling, however, given the shared Wnt/Fzd interaction that is seen in both pathways, as well as the crosstalk that already exists between canonical and non-canonical signalling, it seems likely that Wnt/PCP and Wnt/Ca^{2+} signalling intersect (Grumolato et al., 2010; Hayes et al., 2013).

Interestingly, one of the transcription factors PKCθ is thought to activate is AP-1, in $CD4^+$ T cells (Chirmule, Goonewardena, Pahwa, Pasieka, & Kalyanaraman, 1995). The contribution $CD4^+$ T cells to the pathogenesis of inflammatory bowel diseases (IBD) is currently the focus of a large body of research, and this may provide insight into future intestinal damage models looking at *VANGL2* (Brasseit et al., 2016; Eri, McGuckin, & Wadley, 2012). Further studies should examine Wnt/Ca^{2+} signalling activation to assess *VANGL2*-mediated control of the pathway.

In my *VANGL2* models, I also found several extracellular matrix (ECM) components that are regulated by *VANGL2* in the healthy colonic epithelium. Matrix metalloproteinases (MMPs) are calcium-dependent endopeptidases, which are capable of degrading ECM proteins. MMP2 and MMP9 are both found to be downregulated when *VANGL2* is lost in the epithelium. MMP2 and MMP9 comprise the gelatinase family of MMPs. They are capable of degradation of both gelatin and type IV collagen. Studies show that MMP9 and MMP2 are required for proper remodelling of the ECM at the epithelial basement membrane in the human colon (Z. S. Zeng, Cohen, & Guillem, 1999). This evidence suggests that *VANGL2* promotes the expression of the gelatinase family of MMPs in the colonic epithelium, and that this may act to promote remodelling in the epithelial basement membrane. In human colorectal tumourigenesis, loss of key basement membrane protein type IV collagen is associated with increased expression of both MMP2 and MMP9 (Z. S. Zeng et al.,

1999). Increased expression of MMP2 and MMP9 have also been seen in patients with IBD, and this is also seen in a DSS mouse model of colitis (Lakatos et al., 2012; Sim et al., 2012; Suzuki et al., 2011). Therefore, *VANGL2* may act to predispose the colonic mucosa to disease through gelatinase-MMP basement membrane remodelling. MMP7 is a membrane associated MMP which is known to use laminin, fibronectin, type IV collagen, and gelatin as substrates. It is found to be strongly expressed in areas of mucosal insult (i.e. colitis). Epithelial cells which express MMP7 had disrupted basement membrane, shown by loss of laminin and fibronectin expression (Saarialho-Kere et al., 1996). I revealed that MMP7 expression is upregulated in loss of *VANGL2*, suggesting that *VANGL2* may act to inhibit MMP7 expression in colonic epithelial homeostasis. Tissue inhibitor matrix metalloproteinase 1 (TIMP1) was found to be downregulated when *VANGL2* is lost. In nerve repair, TIMP1 has been shown to be expressed in areas of injury and protects type IV collagen from MMP9-mediated degradation (Fleur, 1996). This suggests that *VANGL2* helps protect areas of injury from ECM breakdown via promotion of TIMP1 expression. If *VANGL2* does indeed protect areas of injury from ECM breakdown, it may play a key role in limiting fibrosis in chronic mucosal injury (e.g. IBD).

The changes in ECM regulators also simultaneously result in changes in the structural components of the ECM, when *VANGL2* is lost in the murine colonic epithelium, such as Proteoglycans (PGs) that fill the interstitial space beneath the

basement membrane. One of these, Decorin, is found to be downregulated in the colonic epithelium when *VANGL2* is lost. SPARC (secreted protein acidic and rich in cysteine) is a glycoprotein that is also downregulated when *VANGL2* is lost. SPARC has previously been implicated in assisting ECM remodelling through promoting collagenase expression (Tremble, Lane, Sage, & Werb, 1993). I showed that a type I collagen precursor, COL1A2, is downregulated when *VANGL2* is lost in the epithelium. COL1A2 is an AP-1 transcription factor target and this reduction may be as a result of the downregulation of various AP-1 components seen in the *VANGL2* LOF epithelium (Chung et al., 1996). This reduction in type I collagen was also observed histologically in *VANGL2*-deficient epithelium, suggesting *VANGL2* has an influential role to play in regulation of the ECM. In future studies, more work could be completed to provide a full picture of the changes to the structural proteins of the ECM, as well as exploring MMP activity changes when *VANGL2* is functionally lost. It would also be of interest to look at later timepoints following gene inactivation to see if the observed changes in the ECM accumulate, and if these changes drive phenotypic changes in colonic epithelial homeostasis.

It may be that this *VANGL2*-mediated ECM turnover results in more profound changes in epithelial tissue. Extracellular matrix (ECM) proteins have been implicated in regulating epithelial-mesenchymal transition (EMT), and I found that EMT marker α-SMA is upregulated in *VANGL2*-deficient epithelial cells (Q. K. Chen, Lee, Radisky,

& Nelson, 2013). As previously discussed, I revealed that ROCK1 and ROCK2 were upregulated in the epithelium when *VANGL2* is lost. Both proteins have previously been shown to promote EMT, suggesting that *VANGL2* may act to restrict EMT via suppression of Wnt/PCP ROCK signalling (H. Peng et al., 2016; Ye et al., 2016; X. Zhang et al., 2016). Coyle et al postulated that, having found that *VANGL2* interacts with MMP14 localisation in human fibrosarcoma cells, pathways mediating ECM proteolysis and cell polarity converge to conduct cellular migration (and therefore, could also regulate EMT) (Coyle, Latimer, & Jessen, 2008). The data here suggests *VANGL2* is an important part of this in the colonic epithelium.

6.2 *VANGL2* as a mediator of colorectal cancer

Using human colorectal cancer (CRC) datasets, murine model of intestinal cancer, and adenoma-derived organoid culture, I have identified *VANGL2* as a putative oncogene in CRC. Previously, *VANGL2* has been shown to influence progression of other cancers, such as overexpression indicating poorer prognosis in breast cancer (Puvirajesinghe et al., 2016). It was also shown that *VANGL2* overexpression increases tumour growth, but this can be restricted by inhibition of JNK signalling. This suggests that *VANGL2* supports JNK/cJun signalling to promote tumour development. In CRC cell lines, overexpression of *VANGL2* has been shown to be associated with reduction in proliferation, colony formation, and Wnt/β-catenin signalling, and in humans methylation of *VANGL2* is associated with pro-tumourigenic markers such as higher tumour grade, MSI status, and BRAF mutation (Piazzi et al., 2013). While this represents a discrepancy with the results presented in this thesis, it may be that VANGL2 has a wide range of effects on CRC progression depending on cancer sub-type.

I identified a subset of colorectal cancer patients with high *VANGL2* tumour expression. While these patients do not experience worsened overall survival (OS), they have a significantly reduced disease-free survival (DFS). Following primary CRC

treatment (such as surgical resection of the adenocarcinoma), *VANGL2*-high patients experience quicker time-to-relapse than those patients without high *VANGL2* expression. Typically, colorectal cancers with reduced time-to-relapse typically have higher invasiveness (as tumour spread decreases the effectiveness of surgical resection) and/or metastatic activity (as the cancer is no longer merely locoregional) (Tougeron et al., 2015). I found that higher *VANGL2* expression in patient tumours is associated with grade II tumours (showing poorer differentiation of tumours) over grade I tumours, while it is also associated with lower invasiveness (associated with T1 over T2). Given the results from our cancer mouse model, it appears initially that *VANGL2* has an effect on initiation, and investigation of *VANGL2's* effects on colonic epithelial invasiveness and metastasis is needed. In other cancers *VANGL2* promotes the degradation of the ECM via MMP14 to promote invasion (B. B. Williams et al., 2012; B. Blairanne Williams et al., 2012).

In concordance with what is seen in human CRC, I revealed that my murine intestinal cancer model had no reduction in survival when *VANGL2* is lost. With any loss of an oncogene it may be expected that CRC survival is improved, however it is important to stress that the *APC*-deficient murine model does not model advanced cancer and is limited in duration by anaemia from small intestinal bleeding. I observed that loss of *VANGL2* leads to a significant reduction in colonic tumours; and can, therefore, conclude that *VANGL2* is oncogenic in the context of hyperactivated

Wnt signalling through APC loss. However, loss of *VANGL2* does not affect growth rate of tumours in this model. The evidence found here is interesting as this is the first time *VANGL2* has been implicated in tumour initiation, with previous research implicating *VANGL2* in the progression of CRC (Piazzi et al., 2013). The regulation of the ECM by *VANGL2* may offer answers as to how *VANGL2* modifies tumour initiation. The ECM is involved in the modulation of CRC in early stages (i.e. tumour initiation/growth), as well as in progression of the disease (i.e. invasion/metastasis) (Pickup, Mouw, & Weaver, 2014). *VANGL2* may, therefore, act to drive changes in the ECM to promote tumour initiation while at the same time predispose the colonic ECM to invasion and cancer progression. While my APC-deficient mouse model of intestinal cancer is commonly used to investigate tumourigenic mechanisms, it has some limitations. Intestinal tumourigenesis is not a faithful reproduction from the human due to the prevalence of small intestinal tumours in the mouse, whereas in humans there is predominance of colorectal tumours and small intestinal cancer is comparatively rare (Huels et al., 2015). Also, this model is limited to investigation of tumour initiation as mice succumb to anaemia before tumour progression-related pathology can occur; therefore, this study is limited to understanding the role of *VANGL2* in early tumours. As mentioned previously, examining the role of VANGL2 in models which recreate more advanced tumour stages would help us to define how VANGL2 regulates the invasive or metastatic properties of CRC. For example, loss of the PI3K/AKT regulator PTEN in the APC-deficient context leads to rapid development

of early and advanced invasive adenocarcinomas (Marsh et al., 2008). Examination of the current cancer model with the addition of *PTEN* loss would allow me to assay the effects of VANGL2-deficiency in the invading intestinal adenocarcinoma.

Previous studies looking at *VANGL2* in human fibrosarcoma cells found that *VANGL2* restricts cellular migration and ECM invasion (via MMPs) (Cantrell & Jessen, 2010). This suggests that *VANGL2* influences invasion, and therefore possibly metastasis, in cancer. In my murine intestinal cancer model, I showed that *VANGL2*-deficient colonic tumours have moderate increases in the ECM components DCN, SPARC, and TIMP1. This upregulation is in contrast with normal colonic epithelium, where I find that the loss of *VANGL2* downregulates the expression of these genes, suggesting that *VANGL2*'s ECM-related activity may be modulated by the tumour context. Decorin has been shown suppress tumour initiation, growth and migration (Bi et al., 2008, 2012). SPARC has also been shown to promote tumour initiation, but restrict enterocyte migration (Sansom et al., 2007). While these proteins may seem to have contrasting roles in CRC, this by no means a complete picture of the ECM, and further depiction of the regulation of the ECM by *VANGL2* is needed. In particular, a longer-term analysis would be beneficial due the long half-lives of ECM proteins. Overexpression of TIMP1 has been shown to increase proliferation, metastasis and restrict apoptosis in colon cancer cells (Song et al., 2016). Given the current knowledge, it may be that *VANGL2* promotes tumour initiation via restriction

of SPARC expression, while *VANGL2* may restrict progression of CRC through promotion of Decorin expression. Contrasting again with what is found in healthy epithelium, I found that type I collagen is increased in *VANGL2*-deficient colonic tumours, while another component of the ECM, laminin, is also increased. Type I collagen has a positive association with the stem-ness of human CRC cells, while laminin expression can be lost in malignant cancer cells (Kirkland, 2009; Zapatka et al., 2007). Interestingly, I found that CTGF is upregulated in *VANGL2*-deficient tumours and tumour-derived organoids. CTGF upregulation seems to be a mediator of early tumour development, while also being an inhibitor of invasion and metastasis of CRC (Jacobson & Cunningham, 2012; B.-R. Lin et al., 2011; Been-Ren Lin et al., 2005). The evidence I have accumulated seems to point towards *VANGL2* as a promoter of tumour initiation, but potentially acting as an inhibitor of malignant features of CRC such as invasion and metastasis. It would be possible to determine if *VANGL2* mediates any effects on intestinal cancer progression *in vivo* however for this we would need to develop a model of invasive cancer which invades and is metastatic. A key basement membrane protein not examined in this work the glycoprotein Fibronectin. Quantification of Fibronectin regulation in the *VANGL2* LOF models used here could yield further insight into changes seen in the ECM. By use of *in vitro* cell culture or *ex vivo* organoid assays, future work could focus on assays examining migration and the breakdown of the ECM.

Using colonic adenoma organoid culture, I showed that YAP/TAZ (Hippo) signalling targets BMP4 and WNT5B are down regulated in *VANGL2*-deficient organoids. Studies have shown that WNT5B promotes proliferation and migration, as well as promoting an EMT phenotype in human lung carcinoma cells (Harada et al., 2017; Kato, Hayakawa, Sakurai, Saiki, & Yokoyama, 2014). This technique could also be used to explore downstream Wnt/PCP signalling. Moreover, the intestinal organoid culture system provides an excellent physiological representation of the intestinal epithelium and, in the case of colonic adenoma organoids, of colorectal cancer. There are, however, limitations to the intestinal organoid model. As this is an epithelial culture the cells lack their native microenvironment. This is an issue concerning cellular response to ECM changes induced by *VANGL2*. Interestingly, co-culture with stromal cells has been performed in organoids and could potentially be used here with intestinal organoids (Mondrinos, Jones, Finck, & Lelkes, 2014). Another issue with organoid culture is the absence of immune cells. This is, again, a particularly important problem for examining the ECM as immune cells are important for ECM regulation. A final issue with organoid culture in relation to the ECM is problems with mimicking the *in vivo* biomechanical forces that are exerted upon cells by the ECM, which is important due to the many cellular response that are created from epithelial cells to their microenvironment. However, this can be overcome by engineering the microenvironment with ECM factors to reproduce what is observed *in vivo* (Soen, Mori, Palmer, & Brown, 2006). Intestinal organoid culture offers novel

ways of examining human CRC in culture. For example, human colonic tumour resections could be cultured; both from *VANGL2* high expression and *VANGL2* low expression tumours, and downstream Wnt/PCP signalling could be assayed, as well as experiments defining migratory phenotypes. One could also transplant human tumour organoids onto mice in order to model malignancy of human CRC with differential *VANGL2* expression (Boj et al., 2015). Colorectal cancer-derived organoid biobanks offer an exciting method to bridge the gap between cancer genetics and clinical trials (Van De Wetering et al., 2015). Patient derived organoids with varying *VANGL2* expression status could be investigated for malignant potential using the above methods looking at migration, invasion, and metastasis. CRC patients within the *VANGL2*-high subclass (as identified in chapter 3) experience worsened DFS, could have adenoma resections cultured as organoids and compared to organoids derived from patients with normal *VANGL2* expression. Migration/invasiveness of the organoids can be analysed by measuring the protrusions of the organoids into the basement membrane matrix (e.g. Matrigel or collagen), while investigating metastatic ability of *VANGL2*-high and *VANGL2*-normal tumours via xenograft of patient-derived organoids into mice may explain why we see differences in disease-free survival (X. Li et al., 2014; Nguyen-Ngoc et al., 2014; Shamir, Coutinho, Georgess, Auer, & Ewald, 2016).

6.3 Future Directions

Given the limited amount of studies into the function of *VANGL2* and indeed Wnt/PCP signalling within the context of the intestine, there are several areas still to be investigated. Although some analysis has been carried out in this thesis studying downstream non-canonical Wnt signalling transcriptional targets, more direct quantification of these pathways, looking at Wnt/PCP mediator phosphorylation, is needed to define *VANGL2*s role within the intestinal epithelium and cancer. We can examine activation the JNK pathway through analysis of the active JNK protein, phospho-JNK, which would allow quantification of JNK activation from western blotting. Similarly, the phospho-active form of cJun (downstream target of JNK signalling) could be analysed in this manner. The RhoA signalling molecule could be analysed for activation by utilising a method for precipitating the active form, RhoA-GTP before western blotting for RhoA (Guilluy, Dubash, & García-Mata, 2011). ROCK activity could be measured by measuring the amount of active phosphorylated form of myosin-binding subunit (p-MBS) over total myosin-binding subunit (t-MBS) (Hata et al., 2011). RhoA can also be inhibited by Rhosin, while Y-27632 is a well-known inhibitor of ROCK (Ishizaki et al., 2000; Shang et al., 2012). It would be useful to assess activation of both the RhoA/ROCK and the JNK/cJun pathways in both *VANGL2* WT and LOF WNT5A-treated organoids to elucidate the role *VANGL2* plays in Wnt/PCP signalling. WNT5A treated organoids have been utilised before to assess its effects

on crypt stem cell homeostasis (Miyoshi et al., 2012). Given the interesting results showing that loss of functioning *VANGL2* results in changes to Wnt/Ca^{2+} signalling targets, in the future we could analyse the activity of this pathway through analysis of the activated form of CaMKII (calcium/calmodulin-dependent protein kinase II), p-CaMKII (Y. Li, Ahrens, Wu, Liu, & Dudley, 2011). CaMKII is a key downstream transducer of the Wnt/Ca^{2+} pathway and has been shown to regulate colon cancer proliferation and migration via ERK1/2 and p38 signalling (W. Chen et al., 2017).

As *VANGL2* loss has been implicated here with many changes to the ECM which are associated with invasiveness and metastasis, future work could focus on one the cellular driver of these processes: migration. *VANGL2* has previously been shown to regulate migration in fibrosarcoma cells (Cantrell & Jessen, 2010). Transient knock down using shRNA targeted towards *VANGL2* could be used in human cancer cell lines to assess the impact the gene has on migratory capacity. Migration can be measured by using a wound healing, or 'scratch' assay. A scratch is created on a cell monolayer and migratory cell intrusion into the scratched area is observed over time, replicating cellular migration in wound healing (C.-C. Liang, Park, & Guan, 2007). If these studies prove that *VANGL2* may direct cancer cell migration, *in vivo* analyses could then be utilised to analyse *VANGL2* as a regulator of metastasis. One method that could be used would be performing implantation of either (A) CRC cell lines (pre-treated for knockdown or overexpression of *VANGL2*) cells or (B)

VANGL2 WT or *VANGL2* LOF adenomas derived from my mouse models into the colonic submucosa of mice (Bettenworth et al., 2016). Tumour growth is followed by metastases to the liver and/or peritoneum in this model.

Further work should be done looking at the ECM. Fibronectin is a very important protein in the basement membrane (like laminin), which was not examined in this thesis. It was previously found that high expression of fibronectin in CRC tissues positively correlated with distant metastasis and poorer prognosis in patients, while knockdown in cells restricted cellular proliferation, migration and invasion (Yi et al., 2016). In future studies, the expression levels of fibronectin in *VANGL2* LOF adenomas could be assayed to support the theory that key basement membrane proteins (including laminin) promoting metastasis are upregulated in *VANGL2* LOF tumours.

Diseases other than cancer are affected by ECM composition. Therefore, if *VANGL2* regulates ECM changes in the intestine, it may also regulate other intestinal diseases. Intestinal fibrosis is a common complication of intestinal diseases such as inflammatory bowel disease (IBD) and colitis. Fibrogenesis is triggered by inflammation and results in ECM protein production by intestinal mesenchymal cells, and fibrosis is the aberrant production of these proteins (Ueha, Shand, &

Matsushima, 2012). One of the pro-fibrogenic signals is CTGF, which was revealed to be upregulated in *VANGL2* deficient adenoma organoids. Considering I also identified pro-fibrotic markers (CTGF and type I Collagen) my IHC quantifications, assessment of this factor and other ECM factors in our V2 LOF model in healthy colonic epithelium and colitis would help define how *VANGL2* might be involved in intestinal fibrosis and wound repair.

Upregulation of the markers indicative of epithelial-to-mesenchymal transition (EMT) was also seen in both the VANGL LOF epithelium and *VANGL2* LOF adenoma organoids. Loss of the cell adhesion molecule E-Cadherin loss is considered one of the most fundamental events of EMT (Serrano-Gomez, Maziveyi, & Alahari, 2016). Also, *VANGL2* has been found to regulate E-Cadherin in epithelial cells through internalisation (Nagaoka, Inutsuka, Begum, hafiz, & Kishi, 2015). This suggests that *VANGL2* may act to regulate entry into the EMT developmental programme through control of E-Cadherin. This could be validated by analysing if *VANGL2* LOF organoids lose E-Cadherin expression (when compared to *VANGL2* WT organoids).

6.4 Conclusions

In conclusion:

- I have localised *VANGL2* and other key regulators of Wnt/PCP signalling within the murine colon.
- I have identified cellular activities mediated by *VANGL2* in the colon, including non-canonical signalling networks, and the deposition of extracellular matrix (ECM) proteins.
- I have identified *VANGL2* as a modulator of intestinal tumourigenesis in mice, and found that it also is associated with disease-free survival in human colorectal cancer (CRC).

My results offer insight towards the role of *VANGL2* and Wnt/PCP signalling within the mammalian intestinal epithelium in homeostasis and in cancer. It is established that *VANGL2* is an influential factor in intestinal tumourigenesis and human cancer progression and raises questions as to which of the mechanisms *VANGL2* mediates regulates tumour initiation. The interactions shown between *VANGL2* signalling and intra- or extra-cellular changes establish an important framework for future study. By investigating the mechanisms downstream of *VANGL2* signalling (which control such changes), we will be able to reveal opportunities to interfere with colorectal cancer and other intestinal pathologies.

Chapter 7: References

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